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(54) Title: NEWLY IDENTIFIED CHOLINEPHOSPHOTRANSFERASES AND ETHANOLAMINEPHOSPHOTRANSFERASES

(57) Abstract: The present invention relates to polypeptides comprising one or more of the amino acid motifs selected from the group consisting of a sequence with at least 80% identity to any of (a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T (b) C-X-D-X(3)-S-G-H-T (c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H, which transfer phosphocholine and phosphoethanolamine, nucleotide sequences coding for any of these polypeptides and nucleotide sequences complementary thereto, plasmids, vectors and a (micro)organism or cell comprising said nucleotide sequences. Furthermore, the present invention relates to processes to produce cholinephosphotransferases and ethanolaminephosphotransferases like sphingomyelin synthase, ethanolamine phosphorylceramide synthase, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase. The present invention also provides the application of said nucleotide sequences to influence the equilibrium reactions or to develop compounds influencing the equilibrium reactions wherein said transferases are involved and the application of said compounds in medical use. Finally, a process has been provided to isolate candidates for functional genes of a previously unidentified enzyme from a large database by isolating candidates for functional genes of a previously unidentified

transduction and membrane trafficking (Simons and Toomre, 2000; Holthuis et al., 2001). Since the discovery of the 'SM cycle' as a putative signaling system analogous to well-known second messenger systems like the phosphoinositide pathway, SM has emerged to the focus of interest in many research laboratories (Kolesnick and Hannun, 1999; Andrieu-Abadie and Levade, 2002).

Since the enzyme SM synthase is also able to catalyse the reverse reaction, namely the formation of PC from SM and DAG (Marggraf and Kanfer, 1984, van Helvoort et al., 1994) it may regulate, in opposite directions, the cellular levels of the bioactive lipids ceramide and DAG. The latter two are important regulators of membrane trafficking, cell proliferation and apoptosis (Kolesnic and Hannun, 1999; Bankaitis, 2002; Brose and Rosenmund, 2002, Pettus et al., 2002). Hence, one may anticipate that the physiological significance of SM synthase goes beyond the formation of SM.

The subcellular localisation of the enzyme has been the subject of numerous studies. After the initial debate had focused on whether SM synthase is located in the Golgi or at the plasma membrane (Marggraf et al.; Voelker and Kennedy, 1982; Lipsky and Pagano, 1985), subsequent work revealed evidence for the presence of SM synthase activity in both membranes (Futerman et al., 1990; Jeckel et al., 1990; van Helvoort et al., 1994). Whether SM synthesis detected at these locations is due to the presence of more than one isoform of the enzyme has remained an open issue.

Progress in understanding the biological roles of SM synthesis and its regulation is hampered by the fact that no successful purification of the responsible enzyme has been achieved. Recent work has identified a bacterial SM synthase released from *Pseudomonas aeruginosa* (Luberto et al., 2003). However, this activity is a soluble protein, hence in contrast to the animal enzyme that is tightly associated with the membrane (Ullman and Radin, 1974; Voelker and Kennedy, 1982). Other efforts focused on the isolation of SM synthase mutants by screening Chinese hamster ovary cells for resistance to a SM-

directed cytolysin (Hanada et al., 1998). Instead of mutants with a primary defect in SM synthase, this method yielded mutants defective in serine palmitoyltransferase activity or blocked in ceramide transport to the site of SM synthesis (Fukasawa et al., 1999).

In the light of the prior art it is therefore highly desirable to identify and purify SM synthases, the composing amino acid sequence and microorganisms producing the same based on a process comprising the expression of nucleotide sequences coding for the amino acid sequences.

Definitions

Throughout this application the following definitions will be used:

- homology: sharing an ancestral molecule
- identity: extent to which amino acid sequences are invariant, in other words unchanged
- "conservative" or "non-conservative" mutation: replacement of one amino acid by another, which does not change the enzyme function upon alignment (lining up sequences to achieve maximum levels of identity and conservation)
- similarity: percentage of identical amino acids or amino acids with similar properties between two sequences.

Abbreviations

Throughout this application the following abbreviations will be used:

Human (Hu)
Caenorhabditis elegans or *C. elegans* (*Ce*)
Drosophila melanogaster (Dm)
Saccharomyces cerevisiae (*Sc*),
Schizosaccharomyces pombe (*Sp*)

Candida albicans (*Ca*)

sphingomyelin (SM)	N-Acyl-D-sphingosine-1-phosphocholine
sphingomyelinase (SMase)	
phosphatidyl choline (PC)	1,2-Diacyl-sn-glycero-3-phosphocholine
diacyl glycerol (DAG)	sn-1,2-Diacyl glycerol
ceramide (CER)	N-Acyl-D-sphingosine

inositol phosphorylceramide (IPC)

lipid phosphate phosphatase (LPP)

N-(7-(4-nitrobenxo-2-oxa-1,3-diazole)aminocaproylsphingosine (NBD-CER)

N-ethylmaleimide (NEM)

electrospray ionization tandem mass spectrometry (ESI-MS/MS)

phytoceramide-based SMs (phytoSM)

phosphorylcholine (Ch-P)

cytidine-5' diphosphocholine-choline (CDP-Ch)

tubovesicular membranes (TVM)

phosphatidylethanolamine (PE)

phosphatidylinositol (PI)

phosphatidylserine (PS)

phosphatidic acid (PA)

phosphatidylglycerol (PG)

open reading frame (ORF)

CSS1, CSS2 and CSS3 (for candidate SM synthase family 1-3),

-- with human CSS3α1 and CSS3α2 renamed as *hu*SMS1 and *hu*SMS2 and C. *elegans* CSS3α1*1* and C. *elegans* CSS3α2 renamed as *ce*SMS1 and *ceSMS2*

-- with human CSS3β renamed as *hu*SMSr (SMS related), with mice CSS3β renamed as *mo*SMS1, with *C. elegans* CSS3β as *ce*SMS1 and *Drosophila* CSS3β, as *do*SMS1
-- with *C. elegans* CSS3γ renamed as *ce*SMSdr, (SMS distantly related).

Description of the figures with legends.

Fig. 1. Selection and phylogenetic analysis of candidate SM synthases. (A) Animal entries in SwissProt/TrEMBL were searched for the presence of a sequence motif shared by LPPs and Aur1p proteins and then further selected on the basis of three additional criteria, as indicated. (B) Phylogenetic tree of human candidate SM synthases (CSS) and previously characterised members of the human LPP super family. (C) Phylogenetic tree of CSS proteins from human (*Hu*), mouse (*Mm*), *Caenorhabditis elegans* (*Ce*) and *Drosophila melanogaster (Dm)*, and of Aur1p proteins from *Saccharomyces cerevisiae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*) and *Candida albicans* (*Ca*). Asterisks denote CSS proteins expressed in *S. cerevisiae* and tested for SM synthase activity. SM synthases (SMS) are marked in red. SwissProt/TrEMBL accession numbers of CSS proteins are: (1) Q9TYV2; (2) Q20696; (3) Q9VS60/Q9VS61; (4) Q96LT4; (5) Q9DA37; (6) Q86VZ5; (7) Q8VCQ6; (8) Q8NHU3; (9) Q9D4B1; (10) Q9XTV2; (11) Q20735; (12) Q965Q4; (13) Q9D606; (14) Q9NXE2; (15) Q8VCY8; (16) Q96GM1; (17) Q96MP0; (18) AAP57768; (19) Q9BQF9; (20) AAP57767; (21) Q22250; (22) Q9TXU1; (23) Q9VNT9; (24) Q9VNU1; (25) Q10022; (26) Q9D4F2; (27) Q8IY26; (28) Q91WB2; (29) Q96SS7; (30) Q8T8T9; (31) Q22461. Note that (4) Q96LT4 contains a partial protein sequence and that the complete ORF was deduced from a corresponding EST clone (see the Table).

Fig. 2. A subset of CSS3 family members displays SM synthase activity upon expression in yeast. **(A)** Immunoblots of cells expressing various CSS proteins were stained with antibodies recognising the V5 epitope-tagged carboxy termini of CSS proteins. Control denotes cells transformed with empty vector. **(B)** TLC separation of reaction products generated when NBD-ceramide (NBD-Cer) was incubated with lysates of control or CSS-expressing cells in the presence (+) or absence (-) of IPC synthase inhibitor, aureobasidin A (Aba). **(C)** Metabolic labelling of cells expressing human CSS3α1/SMS1 or CSS3α2/SMS2 with [^{14}C]-choline and NBD-ceramide. The lipids were extracted, separated by two-dimensional TLC and analysed for fluorescence and radioactivity. Note that SMS1- and SMS2-expressing cells, but not control cells, synthesized NBD-SM, and that this NBD-SM was labelled with [^{14}C]-choline. **(D)** Metabolic labelling of cells expressing human CSS3α2/SMS2 with [^{14}C]-choline. The lipids were extracted, deacylated by mild alkaline hydrolysis (+NaOH) or control incubated (-NaOH) and separated by two-dimensional TLC before autoradiography. Note that SMS2-expressing cells synthesized alkaline-resistant species of [^{14}C]-choline-labelled lipids (phytoSM) that were absent in control cells.

Fig. 3. NEM inhibits yeast-associated SMase but not mammalian SM synthase. **(A)** TLC separation of reaction products generated when NBD-SM (1.8 μM) was incubated with lysates of exponentially grown yeast cells (20 OD_{600} cells) in the presence (+) or absence (-) of *N*-ethylmaleimide (NEM; 1 mM). Reactions were in 0.35 ml RB1 buffer containing 1 μg/ml of IPC synthase inhibitor (aureobasidin A) and incubated for 30 min at 37°C. **(B)** TLC separation of reaction products generated when NBD-ceramide (NBD-Cer; 1,8 mM) was incubated with lysates of HeLa cells (1/1: 2 x 10^6 cells; 1/4: 0.5 x 10^6 cells) in the presence (+) or absence (-) of NEM (1 mM). Reactions were in 0.35 ml PNS buffer, and incubated for 30 min at 37°C. Lipid extraction and TLC analysis were as described in Materials and methods.

Fig. 4. Detergent extracts of human CSS3α2/SMS2-expressing yeast cells support SM formation from bovine brain ceramides. Lines **A** and **B** show the reconstituted ion chromatograms of m/z 731.6, corresponding to the m/z ratio of protonated 18:0 SM, during the separation of molecular species of choline-containing phospholipids after solid phase extraction of membrane extracts that had been incubated with bovine brain ceramides and egg PC. The elution time of an authentic standard of 18:0 SM is indicated in the chromatogram by an arrow (42.3 minutes). Line **A** was derived from detergent extracts of human CSS3α2/SMS2-expressing cells and line **B** from detergent extracts of control cells. Mass spectra recorded at the elution time of 18:0 SM confirmed that formation of 18:0 SM occurred in CSS3α2/SMS2-containing extracts (top right panel), but not in control extracts (bottom right panel).

Fig. 5. SM synthase activity in detergent extracts of human SMS1- or SMS2-expressing yeast cells is stimulated by externally added PC. Detergent extracts of yeast cells expressing human SMS1 or SMS2 were diluted 2- to 8-fold in extraction buffer, and then incubated with NBD-ceramide (1.8 μM) in the presence (+) or absence (-) of externally added PC (22 μM), as described in Materials and methods. Formation of NBD-SM was monitored by one-dimensional TLC.

Fig. 6. Human SMS1 and SMS2 function as PC:ceramide choline phosphotransferases. (**A**) Detergent extracts of yeast cells expressing human SMS1, SMS2 or transformed with empty vector (control) were incubated with NBD-ceramide (1.8 μM) in the presence or absence of different potential head group donors (22 μM), as indicated. Formation of NBD-SM or NBD-IPC was monitored by one-dimensional TLC and quantified as described in Materials and methods. Note that addition of PI stimulated formation of NBD-IPC in all three extracts (asterisks). PC, phosphatidylcholine; SM, sphingomyelin; Ch,

choline; Ch-P, phosphorylcholine; CDP-Ch, cytidine-5' diphosphocholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol. (B) Detergent extracts of SMS2-expressing and control cells were incubated with NBD-ceramide and [^{3}H]-choline labelled PC. The lipids were extracted, separated by two-dimensional TLC and analysed for fluorescence and radioactivity. Note that only SMS2-expressing cells synthesized NBD-SM, and that this NBD-SM was labelled with [^{3}H]-choline.

Fig. 7. Human SMS1 and SMS2 exhibit reverse activity. Detergent extracts of yeast cells expressing human SMS1, SMS2 or transformed with empty vector (control) were incubated with NBD-diacylglycerol (NBD-DAG; 1.8 μM) in the presence or absence of different head group donors (22 μM), as indicated. Formation of NBD-PC was monitored by one-dimensional TLC and quantified as described in Materials and methods.

Fig. 8. Conserved sequence motifs in SMS1, SMS2 and SMSr proteins. (A) Alignment of human SMS1, SMS2 and SMSr amino acid sequences. Identical residues are highlighted in black and conservative amino acid substitutions in grey. Conserved residues within four homology motifs, designated D1 to D4, are highlighted in blue and conservative amino acid substitutions in the D2 and D4 motifs of SMSr proteins in green. Note that motifs D3 and D4 display similarity to the C2 and C3 domains in LPPs, with identical residues highlighted in red. Regions predicted to form transmembrane domains, TM1 to TM6, are marked by a black line. (B) Alignment of the four homology motifs in SMS and SMSr proteins from humans (*Hu*), *C. elegans* (*Ce*), *P. falciparum* (*Pf*) and *D. melanogaster* (*Dm*). PlasmoDB accession no. of *Pf*SMS1 and *Pf*SMS2 are MAL6P1.177 and MAL6P1.178, respectively. Putative active site residues in consensus

sequences are underlined. Symbols used are n, small neutral amino acid; Φ, aromatic amino acid; b, branched amino acid; x, any amino acid.

Fig. 9. Sequence listings 1 - 11: conserved sequence motifs of phosphotransferases as indicated in figure 8 B

Sequence listings 12 - 22: polypeptide sequences of cholinephosphotransferases and ethanolaminephosphotransferases

12. *human* sphingomyelin synthase 1
13. *human* sphingomyelin synthase 2
14. *human* sphingomyelin synthase related
15. *Caenorhabditis elegans* sphingomyelin synthase 1
16. *Caenorhabditis elegans* sphingomyelin synthase 2
17. *Caenorhabditis elegans* sphingomyelin synthase 3
18. *Caenorhabditis elegans* sphingomyelin synthase distantly related
19. *Caenorhabditis elegans* sphingomyelin synthase related
20. *Plasmodium falciparum* sphingomyelin synthase 1
21. *Plasmodium falciparum* sphingomyelin synthase 2
22. *Drosophila melanogaster* sphingomyelin synthase related.

Fig. 10. Human SMS1 and SMS2 are encoded by ubiquitously expressed genes. Northern blot analysis of SMS1 and SMS2 transcripts (arrows) in various human tissues. Random prime-labelled human SMS1 or SMS2 cDNA was hybridised to a human poly(A)$^+$ RNA blot (Origene, Rockville, MD). As a control for loading, the RNA blot was stripped and rehybridized with a human ß-actin cDNA probe. Mobilities of RNA size markers are indicated.

Fig. 11. Human SMS1 and SMS2 localise to different cellular organelles. (A)

HeLa cells co-transfected with V5-tagged SMS1 and *myc*-tagged sialyltransferase were incubated in the presence or absence of 10 μM nocodazole for 60 min at 37°C, fixed, and then co-stained with rabbit anti-V5 and mouse anti-*myc* antibodies. Counterstaining was with FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies. (B) HeLa cells transfected with V5-tagged SMS2 were biotinylated on ice, fixed and then co-stained with mouse anti-V5 and rabbit anti-biotin antibodies. Counterstaining was with Texas red-conjugated goat-anti mouse and FITC-conjugated goat anti-rabbit antibodies. Bar, 10 μm.

Fig.12. Human SMS2 is partially associated with the Golgi. HeLa cells co-transfected with V5-tagged SMS2 and *myc*-tagged sialyltransferase were incubated in the presence or absence of 10 μM nocodazole for 60 min at 37°C, fixed, and then co-stained with rabbit anti-V5 and mouse anti-*myc* antibodies. Counterstaining was with FITC-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies. Bar, 10 μm.

Fig. 13. Membrane topology of human SMS1 and SMS2 proteins. (A) Schematic view of the predicted membrane topologies of V5-tagged SMS1 and SMS2, and the Golgi-associated type I membrane protein, p24. (B) Immunoblot of intact or lysed HeLa cells expressing V5-tagged SMS1 or SMS2 and pretreated with 8 mM trypsin for 30 min at 30°C in the presence or absence of 0.4% Triton X100, as indicated. Immunoblots were stained with antibodies against the V5 epitope (α-V5) or against p24 (α -p24).

Fig. 14. SMS1 and SMS2 are required for cell growth and survival. (A) SMS1, SMS2 and SMSr are expressed in human HeLa cells. Open reading frames of human SMS1, SMS2 and SMSr were amplified by reverse-transcriptase PCR on 5 μg total RNA isolated from HeLa cells, according to

instructions of the manufacturer (Titan One Tube RT-PCR, cat. No. 1855476, Roche Diagnostics GmbH, Mannhein, Germany). Products were separated on a 1% agarose gel and stained with ethidium bromide. (B) HeLa cells stably transfected with V5-tagged SMS1 (SMS1-V5/pcDNA3.1) were treated with lamin A, SMS1 or SMS2 siRNA duplexes for 72 hr before Western blot analysis using anti-V5 and anti-lamin A antibodies, as described (Elbashir et al., Nature 411, 494-498). Lamin A, SMS1 and SMS2 were targeted with siRNA duplexes designed from the sequences 5'-aactggacttccagaagaacatc-3', 5'-aactacactcccagtacctgg-3', and 5'-aacccaagagcttatccagtg-3', respectively. (C) Hela cells stably transfected with V5-tagged SMS1 were treated with lamin A or SMS1 siRNA for 72 hr, and then fixed, permeabilized and co-stained with mouse-anti GM130 and rabbit anti-V5 antibodies. Counterstaining was with Texas red-conjugated goat-anti-mouse and FITC-conjugated goat-anti-rabbit antibodies. (D) Growth curves of Hela cells stably transfected with V5-tagged SMS1 and treated with lamin A, SMS1 and/or SMS2 siRNA duplexes in serum-free Optimem I medium supplemented with Glutamax I (GIBCO/Invitrogen, Carlsbad, CA). Cell numbers were assessed at 0, 24, 48 and 72 hr after treatment with siRNA duplexes.

Table: EST clones and primers used in this study

Protein name	Accession number[1]	EST clone ID	Primer pairs N- and C-terminal (5'-3')
*Hu*CSS1α1	Q9NXE2	IRAKp961F14 32[2]	AAGCCATGGCTGTAGGAAACAACAC GGTAACTTCGGTCATGGAC
*Hu*CSS1α2	Q96GM1	IRALp962F05	AAGCCATGGCGGGAGGGAGA

		26[2]	GGTGGCCACGGCGGGC
*Hu*CSS2 α	Q96SS7	IRALp962I04	AAGCCATGCCAGCTTCCCAGAG
		17[2]	CCAGGCAGAGATGAGCATC
*Hu*CSS3α1	Q86VZ5	LGN01891[3]	AAGCCATGATCCTTGTAGGACTCTG
(SMS1)			TGTGTCATTCACCAGCCGG
*Hu*CSS3α2	Q8NHU3	IRAKp961F14 33[2]	AAGCCATGAATATGTTTGATGCTGACA
(SMS2)			ATCCTTATAAGCCCGTGTGG
*Hu*CSS3β	Q96LT4	IMAGp958I13 1239Q[2]	AAGCCATGGCAGGTCCTAATCAACTC
			TCCAATTAGTCTTTTCATTATTGC
*Mm*CSS1α2	Q8VCY8	IRAKp961L15	AAGCCATGGCTGGAGGGAG
		31[2]	GGTGGCCACGGCGGGC
*Mm*CSS2α	Q91WB2	IMAGp998B0	AAGCCATGCCAGCTTCCCAGA
		810812[2]	CCAGGCAGAGATGAGCATC
*Mm*CSS3α1	Q8VCQ6	IRAKp961L21	AAGCCATGAAGGAAGTGGTTTAC
(SMS1)		41[2]	TGTGTCGTTTACCAGCCGG
*Ce*CSS1γ	Q22250	YK289g7	AAGCCATGTCCGTGCCAGCTTCG
		/YK572h5[4]	GTACCGATATCCATCATTTTG
*Ce*CSS3α1	Q9XTV2	YK559h9 /	AAGCCATGAAAATGTCTTGGAATCATCA
(SMS1)		YK517e10[4]	A TTTTGGCAGAGACATGGTAG
*Ce*CSS3α2	Q20735	YK428e6 /	AAGCCATGACAAACAGTTCGGAGTTC
(SMS2)		YK109h8[4]	TTGCAATTTGTAGTTGATACGA
*Ce*CSS3β	Q20696	YK524b8[4]	AAGCCATGCTGGATAACAGACCTATAC
			ATTGTGCTTTTTGGTATGATTTTTG

1 Swiss-Prot/TrEMBL

2 Obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany

3 Provided by Dr. Sumio Sugano, Institute of Medical Science, University of Tokyo, Japan

4 Provided by Dr. Yuji Kohara, National Institute of Genetics, Mishima, Japan

To achieve the targets of the invention a complementary approach was pursued for the identification of animal SM synthase that takes advantage of structural information available for enzymes catalyzing analogous reactions. In contrast to most animal cells, plants, fungi and yeast do not produce SM. Instead, these organisms add phosphoinositol to phytoceramide to generate inositol phosphorylceramide, or IPC (Dickson, 1998). IPC production in the yeast *Saccharomyces cerevisiae* requires the product of the *AUR1* gene (Nagiec et al., 1997).

Sequence analysis of Aur1p proteins from different fungi revealed four conserved motifs (Heidler and Radding, 2000) two of which are similar to the C2 and C3 domains present in members of the lipid phosphate phosphatase (LPP) family (Waggoner et al., 1999).

LPPs play a critical role in cell signaling by controlling the conversion of bioactive lipid phosphate esters such as (lyso)phosphatidic acid and sphingosine-1-phosphate to their dephosphorylated counterparts. The conserved C1, C2 and C3 domains in LPPs likely constitute the active site for cleavage of the bond between the lipid hydroxyl and phosphate groups (Neuwald, 1997).

In the case of Aur1p, this reaction would represent the first step in the transfer of inositol phosphate from PI, with the resulting enzyme-phosphate intermediate being subjected to nucleophilic attack by the oxygen of ceramide

rather than the oxygen of water used by LPPs. The presence of LPP-like motifs, together with the IPC synthase activity found associated with affinity-purified Aur1p, suggests that Aur1p is directly responsible for IPC synthesis.

Insects and mammals contain an ethanolamine phosphorylceramide (EPC) synthase, another phosphotransferase, that may be structurally related to SM synthase.

The insect *Drosophila melanogaster* (*Dm*) does not generate SM, but instead produces phosphoethanolamine phosphorylceramide (EPC (Rietveld et al., (17). Moreover, it has been shown that the endoplasmic reticulum of mammalian cells harbours an EPC synthase (Malgat et al., 1987). Furthermore, the nematode *Caenorhabditis elegans* (*Ce*) generates phosphorylcholine-substituted glycolipids (Lochnit, G., et al., 2000) still another phosphotransferase, that may be structurally related to SM synthase.

Based on the above considerations a bioinformatics and functional cloning strategy was developed to identify the enzyme responsible for SM synthesis in animals and to identify other cholinephosphotransferases. The present invention provides a surprisingly successful strategy which comprises a process to isolate candidates for functional genes of a previously unidentified enzyme with known activity from a huge database by combining at least four characteristics based on data from bio-informatics and from biochemistry, viz.

– presence of a sequence motif shared with previously identified enzymes having a related function

– biochemical function of the gene should be unknown until now

– no structural homologues in an organism that does not contain the enzyme

– ability to mediate a reaction catalysed by the unidentified enzyme upon its heterologous expression in an organism or cell lacking said enzyme activity.

Preferably also the presence or non-presence of transmembrane domains depending on the working mechanism of the enzyme in relation to the membrane is considered.

By searching the human, mouse, *C. elegans* and *Drosophila* protein databases using a sequence motif shared by LPPs and Aur1p homologues and further selection on the basis of the three additional criteria mentioned above and, a set of cholinephospho transferase sequences was collected as depicted in Fig. 8B. Conserved residues within a lot of homology motifs have been found.

Therefore, the present invention further provides an isolated polypeptide comprising one or more of the amino acid motifs selected from the group consisting of a sequence of at least 80%, preferably 90 % identity to any of

(a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T

(b) C-X-D-X(3)-S-G-H-T

(c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H.

The wording isolated means that said polypeptide has been identified in a database comprising a large amount of polypeptides.

In particular said polypeptide is derived from the group consisting of *Animalia, Alveolata* and *Kinetoplastida*. Preferably, said polypeptide is selected from the group comprising any of the SEQ ID No. 1 - 11 or at least 70%, more preferably at least 80%, still more preferably at least 90% similarity to the conserved amino acid motifs listed therein.

Furthermore the present invention provides a polypeptide comprising an amino acid sequence with at least 70%, preferably at least 80% and still more preferably at least 90% similarity to any of the SEQ ID No. 12 - 22. Each of these sequences comprise amino acid motifs of the SEQ ID No. 1 - 11 or sequences related thereto so that the similarity grade of at least 70 % to any of the SEQ ID No. 12 - 22 will be reached.

The present invention also provides a polypeptide comprising an amino acid sequence with at least 20 % identity to any of the SEQ ID No. 12 - 22. In particular, a polypeptide comprising an amino acid sequence with at least 30 % identity to SEQ ID No. 12 and therefore at least also including SEQ ID No. 13. Furthermore, a polypeptide is provided by the present invention comprising an amino acid sequence with at least 40 % identity to any of the SEQ ID No. 19 - 21. Finally, the invention provides a polypeptide comprising an amino acid sequence with at least 22 % identity to SEQ ID No. 22.

According to a preferred embodiment of the present invention a polypeptide is provided with one or more of the activities selected from the group consisting of cholinephosphotransferases and ethanolaminephosphotransferases, preferably one or more of the activities selected from the group consisting of sphingomyelin synthase, ethanolamine phosphorylceramide synthase, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase. Furthermore, a nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences of a polypeptide with one or more of said activities and an anti sense nucleotide sequence that is complementary thereto is provided, together with a plasmid, a vector and an organism or micro organism and a cell line comprising said nucleotide sequences.

According to a further preferred embodiment of the present invention a process is provided for producing sphingomyelin synthase or sphingomyelin comprising the expression of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for such a polypeptide. Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase activity to influence the reaction

$$CER + PC \leftrightarrow SM + DAG$$

in *vivo* or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of CER and PC into SM and DAG and vice versa. Besides this, the present invention also provides the use of one of more of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase activity to identify or develop compounds influencing the reaction

$$CER + PC \leftrightarrow SM + DAG.$$

According to still a further preferred embodiment of the present invention a process is provided for producing ethanolamine phosphorylceramide synthase or ethanolamine phosphorylceramide comprising the expression of the nucleotide sequences coding for a polypeptide with ethanolamine phosphorylceramide synthase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for such a polypeptide. Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with ethanolamine phosphorylceramide activity to influence the reaction

$$CER + PE \leftrightarrow EPC + DAG$$

in *vivo* or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of CER and PE into EPC and DAG and vice versa. Besides this, the present invention also provides the use of one of more of the nucleotide sequences coding for a polypeptide with ethanolamine phosphorylceramide synthase activity to identify or develop compounds influencing the reaction

$$CER + PE \leftrightarrow EPC + DAG.$$

According to another preferred embodiment of the invention the application of the compounds mentioned above which can be identified or developed by the use of one of more of the nucleotide sequences coding for a polypeptide with sphingomyelin synthase and/or ethanolamine phosphorylceramide synthase in medical use, especially for the manufacture of medicaments treating a disease selected from the group consisting of cancer, metabolic diseases, for instance the Niemann Pick A disease and diseases caused by parasites, for instance malaria, is provided.

According to a further preferred embodiment of the present invention a process is provided for producing phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase, phosphorylcholine-substituted glycoprotein or phosphorylcholine-substituted glycolipid comprising the expression of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase activity in a (micro)organism or cell line comprising the nucleotide sequences coding for the corresponding polypeptide. "Corresponding" stands for the nucleotide sequences which code for the enzyme with the corresponding activity, so either for the enzyme with the phosphatidyl:glycoprotein cholinephosphotransferase activity or for the enzyme with the phosphatidyl:glycolipid cholinephosphotransferase activity. Furthermore, the use of one of more of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase activity to influence the reaction

glycolipid/protein + PC $\leftrightarrow$ PC-substituted glycolipid/protein + DAG

in vivo or *in vitro* has been provided. In this way a powerful means has been created to enhance the conversion of glycolipid/protein and PC into PC-substituted glycolipid/protein and DAG and vice versa.

Besides this, the present invention also provides the use of one of more of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase activity or phosphatidyl:glycolipid cholinephosphotransferase activity to identify or develop compounds influencing the reaction

glycolipid/protein + PC ↔ PC-substituted glycolipid/protein + DAG.

According to another preferred embodiment of the invention the application of the compounds mentioned above which can be identified or developed by the use of one of more of the nucleotide sequences coding for a polypeptide with phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase in medical use, especially for the manufacture of a medicament treating a disease caused by parasitic nematodes, is provided.

A detailed description of the various aspects of the present invention will be given here below.

A novel family of integral membrane proteins likely responsible for SM synthesis in animal cells was identified according to the process of the present invention. The two human members of this family, SMS1 and SMS2, were subjected to a detailed biochemical analysis and it is believed that their functional assignment as SM synthases is justified on the following grounds.

First, heterologous expression of SMS1 or SMS2 proves sufficient to support SM synthesis in the yeast *S. cerevisiae*, an organism lacking endogenous SM synthase activity (figures 2, 4 and 6). Second, in keeping with the enzymatic properties of mammalian SM synthase reported in the

literature, SMS1 and SMS2 function as bi-directional lipid cholinephosphotransferases capable of converting PC and ceramide to SM and DAG and vice versa (figures 6 and 7). Third, SMS1 and SMS2 localize to cellular organelles previously established as the principal sites of SM synthesis, namely the Golgi (SMS1) and the plasma membrane (SMS2), figure 11. Fourth, SMS1 and SMS2 share sequence motifs containing putative active site residues with lipid phosphate phosphatases and candidate PI:ceramide inositolphosphotransferases (figure 8). Finally, SMS1 and SMS2 adopt a membrane topology whereby the putative active site residues are facing the exoplasmic leaflet (figure 13), hence the side of the membrane where SM synthesis was found to occur.

A multiplicity of SM synthase genes appears widely spread among organisms generating SM. Human SMS1 and SMS2 are highly conserved in mammals and evidence was found for the existence of up to three functional homologues in the nematode *C. elegans*. Moreover, two orthologous sequences in the human malaria parasite *P. falciparum* were identified according to the process of the present invention, suggesting that this lower eukaryote too contains different SM synthases encoded by separate genes. SM synthesis in *P. falciparum* occurs in the Golgi apparatus as well as in the network of tubovesicular membranes (TVM) emerging from the parasitophorous vacuolar membrane during intraerythrocytic development (Elmendorf and Haldar, 1994). A differential sensitivity to 1-phenyl-2-acylamino-3-morpholino-1-propanol has been taken as evidence that the Golgi- and TVM-associated activities correspond to different enzymes (Kauer et al., 1995). Our current findings provide a novel opportunity to test this prediction and may facilitate the development of new drugs against parasites as for instance malaria.

The uniform tissue distributions of transcripts for SMS1 and SMS2 in humans (figure 10) suggest that most mammalian cell types would contain both SM synthase isoforms. Biochemical analysis of human SMS1 and SMS2 thus far revealed no fundamental differences in enzymatic properties, even

though the possibility that substrate preferences are lost when enzymes are assayed in a heterologous, detergent-containing system cannot be excluded. For now, the most striking difference between SMS1 and SMS2 concerns their subcellular localisation. While SMS1 seems to represent the well-known Golgi-associated SM synthase, SMS2 primarily resides at the plasma membrane. Hence, SMS1 would be proximal to SMS2 with respect to receiving newly synthesized ceramide from the ER. A challenging prospect is that, whereas the Golgi enzyme would be responsible for generating the bulk of cellular SM, the second enzyme may serve a principal role in signal transduction at the plasma membrane. The activation of a sphingomyelinase (SMase) and subsequent liberation of ceramide at the plasma membrane has been recognised as an important signalling event in the regulation of fundamental cellular processes that include cell cycle arrest, differentiation and apoptosis (Kolesnic and Hannun, 1999; Pettus et al., 2002; Andrieu-Abadie and Levade, 2002). By converting ceramide back to SM, plasma membrane-associated SM synthase may attenuate SMase-induced signalling. This reaction would produce as a side product DAG, which is a signalling molecule in its own right (Brose and Rosenmund, 2002).

Hence, the presence of a SM synthase at the plasma membrane complicates a proper understanding of the role of ceramide as a specific signal-transducer.

A current bottleneck for elucidating the role of SM hydrolysis in cellular signalling is the unambiguous identification of the agonist-stimulated SMase. Both neutral and acid SMases have been implicated, but their precise cellular roles in SM hydrolysis and signalling remain to be clarified (Andrieu-Abadie and Levade, 2002). Since the plasma membrane-associated SM synthase is capable of catalysing the reverse reaction of SM synthesis (van Helvoort et al., 1994), it is tempting to speculate that it may execute part of the signalling events currently attributed to ligand-induced SMase. The observation that both plasma membrane- and Golgi-associated SM synthases exhibit reverse activity brings up the question of how the directionality of these enzymes is

regulated. One possibility is that the direction of the reaction is primarily determined by the relative concentrations of ceramide and DAG in the membrane. On the other hand, given the biological importance attributed to DAG and ceramide, one may expect the interconversion of these molecules to be subjected to a more elaborate form of control. Indeed, it has been shown that activation of SM synthesis in primary astrocytes is an early event associated with the mitogenic activity of basic fibroblast growth factor (Riboni et al., 2001) and that the onset of TNFα-induced apoptosis in rhabdomyosarcoma cells is preceded by TNF-dependent inhibition of SM synthesis (Bourteele et al., 1998). Mammalian SMS1 contains a predicted SAM (sterile alpha motif) domain at the amino-terminus (residues 7-63 in human SMS1) that might provide a means for the enzyme to interact with regulatory proteins (Schultz et al., 1997). The present identification of a family of animal SM synthases offers unprecedented opportunities to further clarify the biological significance of SM metabolism and its regulation.

SM synthases contain four highly conserved sequence motifs (figure 8). Two of these, D3 and D4, are similar to the C2 and C3 phosphatase domains in LPPs and include the putative catalytic histidine and aspartate residues implicated in LPP-mediated hydrolysis of lipid phosphate esters (Neuwald, 1997) This suggests a working mechanism for SM synthases in which the catalytic triad previously described for LPPs would function in a similar fashion. The reaction would proceed via the following steps: (1) binding of a two-chain choline-phospholipid (PC or SM) to a unique binding site, (2) nucleophilic attack on the lipid-phosphate ester bond by the histidine in D4 assisted by the conserved aspartate in this motif, (3) formation of a cholinephospho-histidine intermediate and release of DAG or ceramide, facilitated by the histidine in D3 acting as a base, (4) nucleophilic attack of the C1-hydroxyl of ceramide or DAG on the cholinephospho-histidine intermediate, assisted by the histidine in D3, (5) release of SM or PC from the active site to

allow another round of catalysis. This model provides a framework for site-directed mutagenesis and kinetic studies to reveal the working mechanism of SM synthases.

There is a striking parallel between animal SM synthase and IPC synthase in yeast. Like human SMS1, the yeast enzyme is Golgi localised and contains the key D4 motif in the Golgi lumen at the start of the last membrane span (Levine et al., 2000). This suggests that animal SM synthase and yeast IPC synthase may have evolved from a common ancestor. In contrast, the SM synthase recently identified from *Pseudomonas aeruginosa* (Luberto et al, 2003) lacks the key motifs of animal SM synthase and is a soluble, rather than an integral membrane protein. Hence, it appears that SM synthases arose at least twice during evolution.

Database searches using the catalytic site sequences from LPPs yielded, in addition to SM synthases, numerous other proteins containing partially conserved phosphatase motifs. In many cases, the biochemical functions of these proteins are not known.

Given the present findings, the possibility that some of these LPP-like proteins do not serve as phospholipases but instead catalyse novel kinds of synthetic or transphosphatidylation reactions deserves consideration. For example, mammals and insects synthesize ethanolamine phosphorylceramide, a sphingolipid analogous to SM
(Malgat et al., 1986; Rietveld et al., 1999). Whether production of EPC and SM involves a single transferase that accepts both PC and PE as substrates or requires two separate enzymes has remained an open issue. However, *Drosophila* lacks SM and generates only EPC (Rietveld et al., 1999). Our finding that *Drosophila* contains an SMS-related protein, but no SMS proteins, strongly suggests that the SMS-related proteins described in this study function as dedicated EPC synthases. This possibility is currently under further investigation.

Phosphorylcholine (PC), a small haptenic molecule, is found in a wide variety of prokaryotic organisms, i. e. bacteria, and in eukaryotic parasites such as nematodes, as well as in fungi. Linked to parasite-specific glycoprotein glycans or glycolipids, it is assumed to be responsible for a variety of immunological effects, including invasion mechanisms and long-term persistence of parasites within the host. Numerous reports have indicated various effects of PC-substituted molecules derived from parasitic nematodes on signal transduction pathways in B and T lymphocytes, displaying a highly adapted and profound modulation of the immune system by these parasites.

The Nematoda, comprising parasitic and free-living species (e.g. *C. elegans*), can be regarded as promising prototypic systems for structural analyses, immunological studies and biosynthetic investigations. In this context, *Ascaris suum*, the pig parasitic nematode, is an ideal organism for immunological studies and an excellent source for obtaining large amounts of PC-substituted (macro)molecules. *C. elegans*, as a completely genome-sequenced species and expressing parasite analogous PC-substituted structures, together with the possibility for easy *in vitro* cultivation, represents a conceptual model for biosynthetic studies, whereas filarial parasites represent important model systems for human pathogens, especially in developing countries. Current knowledge on the tissue-specific expression of PC epitopes, structural data of glycoprotein glycans and glycosphingolipids bearing this substituent and biological implications for the immune systems of the respective hosts are reviewed by Lochnit et al., 2000. Since the elucidated structures of these compounds are clearly different from those present in the host, the biosynthesis of nematode-specific, PC-substituted compounds would be a promising target for the development of new anthelminthic strategies.

In the present invention it has been shown that modulating the activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases,

phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase (further referred to as the group of enzymes) in a cell has effects on cell growth and/or survival. By lowering the activity in cells, the cells respond by a reduction in cell growth, increased sensitivity toward apoptotic stimuli and/or reduced cell survival. Upregulation of the activity induces cell growth, rejuvenation of the cells and/or a reduced sensitivity toward apoptotic signals. An effect is, of course preferably relative to the behaviour of normal cells. These cells typically have effective but not saturating levels of an activity of an enzyme of the group of enzymes and preferably of a sphingomyelin synthase. The possibility of detecting a change in the activity of an enzyme of the group, that can be correlated to a particular amino acid sequence has led the inventors to methods for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase, said method comprising providing said cell with a nucleic acid encoding a polypeptide according to the invention, contacting said cell with said compound and determining whether said enzymatic activity is modulated. In a preferred embodiment said method comprises detecting whether said activity is inhibited. Thus the invention further provides the use of an inhibitor of a enzyme activity of the group, preferably a sphingomyelin synthase of the invention, as a cell death promoter. Preferably said cell is a parasitic cell or a mammalian tumor cell, preferably a human tumor cell. The compound capable of modulating said enzyme activity displayed by the cell, can act on the level of expression of the enzyme, wherein a higher expression results in upregulation of the enzyme activity displayed by the cell and wherein a lower expression results in downregulation of the enzyme activity displayed by the cell.

Alternatively, the compound can act on the activity of the enzyme in the cell, i.e. the compound can increase the activity of the enzyme and thereby upregulate the enzymatic activity displayed by the cell, or the compound can decrease the activity of the enzyme and thereby downregulate the enzymatic activity displayed by the cell.

In a preferred embodiment of a method of the invention, said enzyme activity of the group comprises a sphingomyelin synthase activity. A polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) or (c). In a preferred embodiment said polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) and (c). In a particularly preferred embodiment a polypeptide of the invention comprising sphingomyelin synthase activity in a mammalian cell (a mammalian sphingomyelin synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with huSMS1 (Seq. ID 12) or huSMS2 (Seq. ID 13) depicted in figure 8. In a particularly preferred embodiment a polypeptide of the invention comprising sphingomyelin synthase activity in a nematode cell (a nematode sphingomyelin synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with ceSMS1 (Seq. ID 14), ceSMS2 (Seq. ID 15) or ceSMS2 (Seq. ID 16) depicted in figure 8. A polypeptide of the invention comprising sphingomyelin synthase activity in a plasmodium cell (a plasmodium sphingomyelin synthase) and having the above mentioned identity to the formulas, preferably further comprises at least 30% and preferably at least 90% sequence identity with pfSMS1 (Seq. ID 17) or pfSMS2 (Seq. ID 18) depicted in figure 8. In a particularly preferred

embodiment a polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in a mammalian cell (a mammalian ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90%, sequence identity with huSMSr (Seq. ID 19). In a particularly preferred embodiment a polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in a nematode cell (a nematode ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, further comprises at least 50% and preferably at least 90% sequence identity with ceSMSr (Seq. ID 21) depicted in figure 8. A polypeptide of the invention comprising ethanolamin phosphorylceramid synthase activity in an insect cell (an insect ethanolamin phosphorylceramid synthase) and having the above mentioned identity to the formulas, preferably further comprises at least 50% and preferably at least 90% sequence identity with dmSMSr (Seq. ID 20) depicted in figure 8. A polypeptide of the invention comprising PC:glycolipid/glycoprotein cholinephosphotransferase activity in a nematode cell (an nematode PC:glycolipid/glycoprotein cholinephosphotransferase) and having the above mentioned identity to the formulas, preferably further comprises at least 30% and preferably at least 90%, sequence identity with ceSMSdr (Seq. ID 22) depicted in figure 8.

In one embodiment of the invention a polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) or (c). In a preferred embodiment said polypeptide of the invention comprising sphingomyelin synthase activity preferably comprises an amino acid sequence having at least 80% and preferably at least 90% identity to an amino acid sequence according to formula (a), (b) and (c). In a particularly preferred embodiment a mammalian polypeptide of the invention comprising sphingomyelin synthase activity and having the above mentioned identity to the formulas further

comprises at least 50% and preferably at least 90%, sequence identity with huSMS1 (Seq. ID 12) or huSMS2 (Seq. ID 13) depicted in figure 8.

By providing a cell with a polypeptide of the invention having an enzyme activity of the group one produces a cell having more activity of said enzyme than before. This allows for early and easier detection of the effect that compounds have on the activity displayed by the cell. In a preferred embodiment a polypeptide of the invention having activity of an enzyme of the group is provided to a cell that did not contain this activity in detectable amounts. Such cells may be generated by inactivating the endogenous genes coding for the polypeptide or cells can be used from species and/or genera that do not contain this activity. Non-limiting examples of such species and/or genera in case of sphingomyelin synthase activity are: drosophila and yeast. Thus in a preferred embodiment the method ulitizes a cell that was deficient in an enzyme activity of the group, which is preferably a sphingomyelin synthase activity, prior to providing the cell with the polypeptide of the invention.

A cell can be any type of cell. A cell can be part of an organism, where one or more of the cells of the organism are provided with (nucleic acid encoding) said polypeptide. The cell can also be a cell of a micro-organism. The micro-organism can be bacterial and is preferably a eukaryotic micro-organism. Yeasts and fungal micro-organisms are preferred.

A polypeptide of the invention preferably comprises a sequence as depicted in figure 8 or a functional part, derivative and/or homologue thereof. A functional part, derivative or homologue of a sequence as depicted in figure 8 comprises at least the same enzyme activity in kind not necessarily in amount. A functional part preferably comprises at least 80% of the amino sequence of a sequence as depicted in figure 8. A functional derivative preferably comprises not more than 5 and preferably not more than 3 amino acid substitutions

compared to a sequence of figure 8. In a preferred embodiment said substitutions are conservative substitutions. A derivative can also comprises one or more deletions and/or insertions of amino acids when compared with a sequence as depicted in figure 8. A functional homologue is a sequence derived from a different species as the species depicted in figure 8, but wherein said homologue shares a common ancestor. A functional homologue preferably comprises an amino acid sequence of formula (a), (b) and (c). In a preferred embodiment said polypeptide comprises a sequence as depicted in figure 8A or a functional part, derivative and/or homologue thereof. In one preferred aspect of the invention said polypeptide is derived from a plasmodium. Preferably having an amino acid sequence as depicted in figure 8B or a functional part, derivative and/or homologue thereof.

The compound can be any chemical or biological compound. In one embodiment said compound has an effect on expression of a polypeptide of the invention in a cell. Such a compound can be an expression cassette comprises an expressible coding region for said polypeptide. It can also comprise for instance a compound capable of modulating the activity of a promoter for the gene encoding said polypeptide in said cell. In a preferred embodiment said compound is capable of preventing translation of mRNA encoding said polypeptide in said cell. Translation is preferably inhibited through anti-sense RNA or RNAi. In a particularly preferred embodiment translation is inhibited through RNAi. RNAi is a collective term for a response of cells to double stranded RNA. A variety of different types of compounds are able to induce the specific degradation of mRNAs. None of those different types of compounds are herein excluded. In a preferred embodiment the compound comprises siRNA (Elbashir et al., Nature 411, 494-498) or a hairpin RNA. A hairpin RNA can be introduced directly into the cell or be expressed by a gene encoding the hairpin.

In another aspect the invention provides the use of a nucleic acid encoding a polypeptide according to the invention, as a probe. In a preferred aspect the invention provides the use of an oligonucleotide specific for a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof, for detecting said sequence.

Detection of the sequence can be for any purpose and is preferably for the purpose of assessing whether a cell comprises an enzyme activity of the group of enzymes mentioned above, preferably sphingomyelin synthase activity.

In another aspect the invention provides use of a nucleic acid encoding a polypeptide of the invention, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof for enhancing cell survival and/or cell growth. It has been found that increasing the enzymatic activity displayed by a cell of an enzyme of the group of enzymes, has the effect that the secretion pathway of the cell is stimulated. This in yet another aspect the invention provides a method for at least in part improving the yield of an secretion product of a cell comprising providing said cell with a polypeptide of the invention, or a nucleic acid such a polypeptide, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof. In a preferred embodiment said polypeptide comprises sphingomylin synthase activity. In this embodiment said cell is preferably a mammalian cell or a cell of a eukaryotic micro-organism.

A method for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases,

phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase can be performed using any method for the detection of said enzymatic activity. In a preferred embodiment the method for detecting said enzyme activity comprises providing said cell or a fraction thereof with a labelled substrate for said enzyme. Said substrate is preferably a substrate for sphingomyelin synthase. The method preferably further comprises harvesting sphingolipid from said cell or said fraction and detecting labelled sphingolipid. Detection of said labelled sphingolipid is preferably done using (thin layer) chromatography or mass spectrometry.

Polypeptides of the invention are associated with a memebrane of the cell. The localization varies with the particular polypeptide. It has been observed that this property can be used to generate chimearic polypeptides having sequences from at least two polypeptides of the invention which have an altered distribution in the cell when compared to at least one of the polypeptides said chimearic polypeptide is derived from. Such chimearic polypeptides and nucleic acids encoding the chimearic polypeptides are therefore also included in the invention. Thus the invention provides a method for targeting a first polypeptide according to the invention to a different cellular compartment comprising providing a cytosolic part of said first polypeptide with a cellular compartment localization signal of a cytosolic part of a second polypeptide according to the invention, wherein said first and said second polypeptide, when unmodified, reside in different cellular compartments. Chimearic polypeptides wherein the at least two original polypeptides reside in essentially the same cellular compartment are also included in the invention. It is also possible to attach known cellular localization signals to a polypeptide of the invention. Such known cellular localization signals, for instance, an endoplasmatic reticulum retention signal do not have to be derived from a polypeptide of the invention. The cellular localization signal is preferably present in the cytosolic C-terminal end of a

polypeptide of the invention. Thus preferably the cytosolic C-terminal end of said first polypeptide is provided with the with a cellular compartment localization signal of a cytosolic part of the second polypeptide. Preferably said cytosolic part of said second polypeptide comprises the C-terminal end of a polypeptide of the invention. Another way to target a polypeptide of the invention to a different compartment is to remove the cellular localization signal from the cytosolic part (preferably the C-terminal cytosolic part) of the polypeptide.

The polypeptide can be targeted to any cellular compartment having a membrane and be active there. In a preferred embodiment said cellular compartment comprises a plasma membrane, an endosomal compartment, a Golgi, an endoplasmatic reticulum or a combination thereof. Cellular localization signals do not have be absolute in that there can be cases wherein detectable amounts of polypeptide are spread over at least two cellular compartments. A polypeptide is said to be targeted to a different compartment when, compared to the original cellular localization of one of the polypeptides of the invention, the chimearic polypeptide is detectable in at least on other compartment or is not detectable in a compartment where one of the single polypeptides is detectable. A special case of targeting is where not the localization but rather the relative distribution over compartments changes. For instance, when an percentage X of a polypeptide of the invention is normally present in a first compartment and a percentage Y is present in a second compartment. In this example targeting is said to have taken place when the percentage in at least one of said at least two compartments is altered by more than 30%. Preferably, the change is reciprocal.

Considering that the cellular compartment localization signal of a polypeptide of the invention is predominantly determined by a C-terminal cytosolic part of said polypeptide it is preferred that said cellular compartment localization signal of the C-terminal cytosolic part of said second polypeptide replace the C-terminal cytosolic part of said first polypeptide.

It will be clear to the skilled in the art that by the present invention yielding purified cholinephosphotransferases and ethanolaminephosphotransferases a new field of development for new compounds with valuable properties as for instance drugs has been made available. The present invention will further be elucidated by the following examples without in any way restricting the broad scope thereof.

Materials and methods

Chemicals

Sphingosyl-(NBD-hexanoyl)-phosphocholine (NBD-SM) and (NBD-hexanoyl)-ceramide (NBD-Cer) were from Molecular Probes (Eugene, OR) and Oleoyl-(NBD-hexanoyl)-phosphocholine (NBD-PC) from Avanti Polar Lipids (Alabaster, AL). [methyl-^{14}C]-choline was from ICN Biomedicals (Irvine, CA) and L-3-phosphatidyl [N-methyl-^{3}H]-choline,1,2-dipalmitoyl from Amersham Pharmacia (Piscataway, NJ). NBD-DAG was produced from NBD-PC by treatment with phospholipase C from B. cereus as described (Trotter, 2000). Egg L-α-phosphatidylcholine, lyso-phosphatidylcholine, L-α-phosphatidylethanolamine, L-α-phosphatidylglycerol, L-α-phosphatidylserine, soybean L-α-phosphatidylinositol, dioctanoyl L-α-phosphatidic acid, SM (from bovine brain), choline, phosphocholine, cytidine 5'-diphosphocholine, O-tricyclo[5.2.1.0^{2,6}]dec-9-yl dithiocarbonate potassium salt (D609), N-ethylmaleimide (NEM) and phospholipase C (Bacillus cereus) were from Sigma Aldrich (St. Louis, MO).

Selection, cloning and expression of CSS sequences

Animal entries in the Swiss-Prot/TrEMBL protein sequence database were searched using Prosite (http://www.expasy.org/tools/scanprosite) against sequence motif H-[YFWH]-X_2-D-[VLI]-X_2-[GA]-X_3-[GSTA] and then further selected based on three additional criteria as described under Results. Candidate SM synthases (CSS) were assembled into different groups by multiple sequence alignments and phylogenetic trees generated with ClustalW and PHYLIP. Open reading frames (ORFs) in selected CSS sequences were PCR amplified using Taq polymerase (MBI Fermentas, Hanover, MD) according to information provided in Table I. PCR products were cloned into yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen Corporation, Carlsbad, CA) and the resulting plasmids used to transform S. cerevisiae strain IAY11 (MATα ura3-52 his3Δ200 leu2-3, -112 trpΔ901 ade2-101 ade3-Δ853, provided by Ian Adams, MRC-LMB, Cambridge, UK). Transformants were grown in complete minimal uracil dropout medium containing 2% (w/v) galactose. Expression of CSS proteins was verified by Western blot analysis with mouse anti-V5 antibodies (Invitrogen). PCR amplified ORFs of human SMS1 and SMS2 were cloned into mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen) and the resulting plasmids (SMS1-V5/pcDNA3.1 and SMS2-V5/pcDNA3.1) used to transfect HeLa cells.

SM synthase assay on cell lysates

CSS-expressing yeast cells were lysed at 100 OD_{600}/ml by bead bashing at 4°C in RB1 buffer (120 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 2 mM $MnCl_2$, 2 mM $MgCl_2$, 20 mM Hepes-KOH, pH 7.2) containing freshly added protease inhibitors (Holthuis et al., (1998).

Lysates were centrifuged at 700 *g* for 10 min at 4°C and supernatants (0.25 ml per reaction) preincubated with NEM (1 mM) for 10 min at 37°C. The reaction was initiated by addition of 5 nmol of NBD-Cer in 0.1 ml RB1 buffer

and incubation continued for 15 min. Lipids were extracted by addition of 1.7 ml chloroform:methanol (1:2.2), dried under N_2 and subjected to butanol/water partitioning. Lipids recovered from the butanol phase were separated by one-dimensional TLC for 30 min in solvent I: chloroform/methanol/4.2 M NH_4OH (9:7:3, v/v), followed by 30 min in solvent II: chloroform/methanol/acetic acid (45:30:7, v/v). Fluorescent images were recorded on a STORM 860 Imaging Analysis System (Molecular Dynamics, Sunnyvale, CA) and analysed with ImageQuant software.

Metabolic labelling

CSS-expressing yeast cells (0.5 OD_{600}) were inoculated in 5 ml medium containing 10 µCi [*methyl*-^{14}C]-choline and 10 nmol NBD-Cer. Cells were grown for 16 h at 30 °C, washed in water, and lipids were extracted by bead bashing in H_2O/methanol/chloroform (5:16:16, v/v). The organic extracts were dried under N_2, subjected to butanol/water partitioning, and lipids recovered from the butanol phase were deacylated by mild base treatment using 0.2 N NaOH in methanol (60 min, 30 °C). After neutralizing with 1 M acetic acid, lipids were extracted with chloroform and separated by two-dimensional TLC using solvent I in the first, and solvent II in the second dimension. Radio-labelled lipids were detected by exposure to BAS-MS imaging screens (Fuji Photo Film Co., Japan) and read out on a BIO-RAD Personal Molecular Imager.

Preparation of detergent extracts

SMS1/SMS2-expressing yeast cells were lysed at 100 OD_{600}/ml by bead bashing at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.3 M sucrose) containing protease inhibitors. Post nuclear supernatants were prepared as above and loaded onto a 60% (w/w) sucrose cushion and then

centrifuged at 100.000 *g* for 60 min at 4 °C. Membranes derived from ± 500 OD_{600} of cells were resuspended in 1 ml ice-cold extraction buffer (50 mM Tris-HCl, pH 7.0, 10% glycerol, 1% Triton X-100, 1 mM $MnCl_2$) containing protease inhibitors, incubated for 60 min at 4 °C while rotating and then centrifuged at 100.000 *g* for 60 min at 4 °C. Detergent extracts were diluted to a final protein concentration of 1.5 mg/ml in extraction buffer and 100 µl aliquots were snap frozen in liquid N_2 and stored at -80 °C.

SM synthase and reverse transferase assays on detergent extracts

Detergent extracts were diluted 5- to 8-fold in extraction buffer, and 50 µl mixed with 5 µl of a 1.2 mM head group donor stock (e.g. CDP-choline, PC, PI) or with 15 µCi (350-700 fmol) of [^{3}H]-choline-containing PC prepared in extraction buffer. Following a 15 min preincubation at 37 °C, reactions were started by addition of 220 µl RB2 buffer (50 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM NEM) containing 5 nmol NBD-ceramide (SM synthase assay) or 5 nmol NBD-DAG (reverse transferase assay) and incubated for 60 min at 37 °C. Lipids were extracted, separated by TLC, and analysed as above.

Mass spectrometry

Detergent extracts (400 µl) were mixed with bovine brain ceramide (180 µM) and egg PC (500 µM) in a total volume of 500 µl. After 15 min at 37 °C, reactions were diluted 4-fold in RB2 buffer and then incubated for another 60 min at 37 °C. Lipids were extracted in chloroform:methanol (1:2.2) and subjected to butanol/water partitioning. Residual Triton X-100 was removed by solid phase extraction on Si-60 columns (500 mg stationary phase) in acetone. Phospholipids were eluted in methanol/chloroform (19:1, v/v), dried under N_2, dissolved in methanol/chloroform (2:1, v/v) and then separated by HPLC on

two 250 x 4.6 mm Lichrosphere RP-18 end-capped columns (Merck, Darmstadt, Germany) in series (Brouwers et al., 1998). The column effluent was split in a 10:1 ratio, with the smaller fraction going to the mass spectrometer. Mass spectrometry was performed on a Sciex API-365 triple quadrupole mass spectrometer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Positive ions were generated by a turbo-ion spray ionisation source operating at +5.5 kV ionisation potential. N_2 (2 l/min) was used as drying gas at a temperature of 350 ° C. The declustering potential (cone voltage) was set to 45V and the focus potential to 220V. Mass spectra from mass to charge ratio (m/z) 650 amu to 950 amu were recorded at a speed of 120 amu/s.

Mammalian cell transfection and immunofluorescence microscopy

HeLa cells were cultured in DMEM medium containing 10% fetal calf serum. Cells were grown on glass coverslips to 40% confluence and then transfected with human SMS1-V5/pcDNA3.1, SMS2-V5/pcDNA3.1 and *myc*-tagged sialyltransferase/pCB7 constructs using Lipofectamine 2000 (Invitrogen). After 48 h, cells were fixed in 3% paraformaldehyde/PBS and processed for immunofluoresence microscopy (see Supplementary materials for details). For cell surface biotinylation, cells were incubated twice with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS for 20 min on ice, quenched in 10 mM glycine/PBS for 20 min and then washed in PBS prior to fixation. Immunostaining was with rabbit anti-V5 antibodies (Sigma), mouse anti-V5 antibodies (Invitrogen), mouse 9E10 anti-*myc* antibody (SanverTech, CA), rabbit anti-biotin antibodies (Rockland, Gilbertsville, PA), FITC- or Texas Red-conjugated goat anti-rabbit and goat anti-mouse antibodies (Jackson Laboratories, West Grove, PA). Images were obtained using a Nikon D-eclipse C1 confocal miscroscope.

Protease protection assay

HeLa cells transfected with human SMS1-V5 or SMS2-V5 constructs were washed, scraped and then lysed in ice-cold PNS buffer (120 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 2 mM $MnCl_2$, 0.8 mM $CaCl_2$, 2 mM $MgCl_2$, 1.6 mM EDTA and 20 mM Hepes-KOH, pH 7.2) by 20 passages trough a $26^3/_4$ gauge needle. The homogenate was treated with 8 mM trypsin (Sigma Aldrich) in the presence or absence of 0.4% Triton X-100 for 30 min at 37 °C. Alternatively, cells were only washed and then trypsin-treated in PNS buffer as above. Homogenates and cell suspensions were transferred on ice, trypsin inhibitor (Sigma Aldrich) was added to 160 mM and samples were processed for Western blot analysis using mouse anti-V5 antibodies and rabbit anti-p24 protein (Gommel et al., 1999, provided by B. J. Helms, Faculty of Veterinary Medicine, Utrecht).

SDS-PAGE and Western Blot Analysis

Protein extracts were prepared in SDS/Urea sample buffer (20 mM Tris-HCl, pH 6.8, 1 mM EDTA, 4.5 M urea, 2.5% SDS, 0.01% bromophenol blue) containing fresh protease inhibitors (Holthuis et al., (1998). Extracts were heated for 10 min at 50 °C, centrifuged briefly at 14,000 *g* and then resolved by SDS-PAGE on 10% polyacrylamide mini-gels. For Western blotting, nitrocellulose transfers were blocked for 90 min in PBS, 5% Protifar (Nutricia, Zoetermeer, The Netherlands), 0.2% Tween-20 (Blotto). Primary antibody incubations were performed for 1 h in Blotto. Detection was with horsheradish

peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence Microscopy

HeLa cells were grown on coverslips, transfected with human SMS1-V5/pcDNA3.1, SMS2-V5/pcDNA3.1 and *myc*-tagged sialyltransferase/pCB7 (Sprong et al., 2001). 48 h after transfection, cells were fixed in 3% paraformaldehyde/PBS for 30 min at room temperature, quenched in PBS containing 50 mM NH_4Cl, and then blocked and permeabilized for 1 h in PBS containing 0.5% BSA, 0.1% saponine (blocking buffer). Cells were labelled with mixtures of primary antibodies (1:200) for 1 h in blocking buffer, washed for 45 min in blocking buffer with three buffer changes, and then incubated for 20 min with 10% goat serum in blocking buffer. For immunostaining of intact cells, incubation with primary antibodies occurred before fixation and was performed for 1 h at 4°C in PBS containing 0.5% BSA. Cells were counterstained with fluorescently labelled secondary goat antibodies (1:75) for 30 min in blocking buffer, washed in blocking buffer for 45 min with three buffer changes, and then rinsed briefly in PBS and water. Mounting was in Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo[2.2.2.]octane (Sigma).

Example 1

Identification of candidate SM synthases from the animal database

Figure 1A shows an outline of the bioinformatics approach used to identify candidate sequences for SM synthases from the animal database. Candidate SM synthases were identified based on the following criteria: 1) presence of a sequence motif, H-[YFWH]-X_2-D-[VLI]-X_2-[GA]-X_3-[GSTA], shared by previously characterised LPPs and Aur1p homologues; 2) biochemical function should be unknown; 3) no structural homologues in S. cerevisiae, since this organism lacks SM; 4) presence of multiple (>2) transmembrane domains, since the enzyme mechanism is intramembranous and because LPPs and Aur1p proteins have 6 predicted membrane spans. Sequences conforming to all 4 criteria were subsequently used as queries in a BLAST search to track down homologous sequences that were missed in the initial search due to deviations in the LPP/Aur1p motif. This approach yielded 9 human, 9 mouse and 9 *C. elegans* sequences that could be grouped into three major protein families, designated CSS1, CSS2 and CSS3 (for candidate SM synthase family 1-3; Figure 1B, C). Except for the presence of a common sequence motif, CSS proteins displayed no significant sequence similarity to Aur1p proteins or to LPP family members with known biochemical functions. However, human CSS1β1 is identical to PRG1, a neuron-specific candidate phosphatidic acid phosphatase with a role in axon growth and regenerative sprouting (Brauer et al., 2003)

Database accession numbers of CSS proteins are listed in the legend of Figure 1.

Example 2

A subset of CSS3 family members displays SM synthase activity

To investigate whether any of the three CSS families indeed contained SM synthases, the open reading frames of human, mouse and *C. elegans* members for which full-length cDNAs could be obtained were cloned into a yeast multicopy, GAL1 promotor plasmid in frame with a carboxy-terminal V5 epitope. The resulting plasmids were used to transform wild-type yeast and the transformants were shifted to galactose-containing medium to induce expression of recombinant proteins. Expression of proteins was verified by Western blot analysis using anti-V5 antibodies (Figure 2A). Thus, 13 of the 27 selected CSS sequences were expressed and analysed for SM synthase activity (marked by asterisks, Figure 1C). To this end, yeast cells expressing CSS protein were lysed and incubated with fluorescent C_6-NBD-ceramide (NBD-Cer), a known substrate of mammalian SM synthase. Incubations were performed in the presence of N-ethylmaleimide (NEM), a potent inhibitor of yeast-associated SMases that does not affect mammalian SM synthase (Figure 3). Reaction mixtures were next subjected to one-phase lipid extraction and the lipids separated by one-dimensional TLC.

Figure 2B shows that in lysates of control cells NBD-Cer was converted exclusively into NBD-IPC. The same was true for lysates of cells expressing members of the CSS1 and CSS2 protein families. However, in lysates of cells expressing human CSS3α1, human CSS3α2, *C. elegans* CSS3α1 or *C. elegans* CSS3α2, NBD-Cer was converted to a second product with an Rf value identical to that of NBD-SM. The identity of this product as NBD-SM was confirmed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS; data not shown). Moreover, metabolic labelling of cells expressing human CSS3α1 or CSS3α2 with [^{14}C]-choline in the presence of NBD-Cer resulted in the production of radio-labelled NBD-SM (Figure 2C). When NBD-Cer was

omitted, we noticed that CSS3α2-expressing cells generated small amounts of [^{14}C]-choline-labelled lipids that were absent in control cells and distinct from PC and lysoPC. In addition, these lipids were resistant to mild alkaline hydrolysis, suggesting that they represent phytoceramide-based SMs (phytoSM; Figure 2D). Importantly, ESI-MS revealed that bovine brain ceramides incubated with detergent extracts of CSS3α2-expressing cells, but not control cells, are converted to SM (Figure 4). Together, these results indicate that CSS3α proteins do not only use NBD-Cer, but also recognise naturally occurring ceramides as substrates for SM synthesis.

Human CSS3α1 and CSS3α2 share 57% sequence identity and are highly conserved in mammals (human – mouse > 90%). Based on the results presented herein, we propose to rename these proteins SMS1 and SMS2, respectively. *C. elegans* CSS3α1 (ceSMS1) and CSS3α2 (ceSMS2) share 22-27% sequence identity with human SMS1 and SMS2, and apparently represent their functional counterparts in the nematode (Figure 2B). *C. elegans* contains a third, CSS3α-related protein, termed ceCSS3α3. Although we have not tested this protein for SM synthase activity, the finding implies that *C. elegans* is equipped with three independent SM synthases (Figure 1C). BLAST searches for orthologous sequences identified two proteins in the human malaria parasite Plasmodium falciparum (PlasmoDB accession no. MAL6P1.177 and MAL6P1.178; see also below). Hence, a multiplicity of SM synthase genes appears a general feature of organisms generating SM.

Apart from CSS3α/SMS proteins, the CSS3 family contains a second cluster of CSS3β or SMS-related (SMSr) proteins with members in *humans*, mice, *C. elegans, and Drosophila* (Figure 1C). *Human* SMSr is highly conserved in mammals (human-mouse: 95%), shares > 40% sequence identity with its orthologues in *C. elegans* and *Drosophila*, and is about 34% identical to human SMS1 and SMS2. Heterologous expression of human or *C. elegans* SMSr did not yield any detectable SM synthase activity (Figure 2B, right panel). The *C. elegans* CSS3γ protein, renamed SMSdr, is only distantly

related to SMS and SMSr proteins (<22% identical; see also Figure 1C) and its ability to mediate SM synthesis was not tested.

Example 3

Human SMS1 and SMS2 function as PC:ceramide cholinephosphotransferases with reverse activity

SM synthesis in animals proceeds by the liberation of phosphorylcholine from PC and its subsequent transfer onto the primary hydroxyl of ceramide. To establish whether human SMS1 and SMS2 function as PC:ceramide cholinephosphotransferases, their enzymatic characteristics were analysed in more detail. Hence, different donors of choline-P were tested as substrates for SM synthesis. To this end, detergent extracts of cells expressing human SMS1 or SMS2 were incubated with NBD-Cer and the formation of NBD-SM was monitored by TLC. A dilution of extracts proved necessary to render SMS-mediated synthesis of NBD-SM dependent on externally added head group donors (Figure 5). Under these conditions, free phosphorylcholine (Ch-P) and CDP-choline (CDP-Ch) did not support SMS1- or SMS2-mediated SM synthesis (Figure 6A). PC, on the other hand, was efficiently recognised as substrate. SM itself was also used as a donor of the phosphorylcholine group. Lyso-PC was a very poor substrate. These results suggest that human SMS1 and SMS2 are transferases that require two fatty chains on the choline-P donor molecule in order to be recognised efficiently as a substrate.

Example 4

The reaction of human SMS1 or SMS2 with non-choline phospholipids

We next investigated the possibility that human SMS1 or SMS2 would use non-choline phospholipids as substrates. None of the phospholipids tested (PE, PI, PS, PA, PG) other than PC supported SM formation (Figure 6A).

Moreover, when extracts of cells expressing SMS2 were incubated with PC containing [^{3}H]-choline, the formation of radiolabelled NBD-SM was observed (Figure 6B). Hence, it appears that SMS1 and SMS2 directly and specifically recognise the choline head group on their substrates.

5

Example 5

The reaction of sphingomyelin and diacyl glycerol

Previous work suggested that mammalian SM synthase is also capable
10 of catalysing the reverse reaction, namely the formation of PC from SM and DAG (Marggraf and Kanfer, 1984; van Helvoort et al., 1994).

To investigate whether this was also the case for human SMS1 and SMS2, extracts of cells expressing these proteins were incubated with NBD-DAG and the formation of NBD-PC monitored by TLC. Addition of SM induced
15 NBD-PC formation in extracts of SMS1- or SMS2-expressing cells, but not in control cell extracts (Figure 7). Strikingly, PC itself proved more efficient than SM in stimulating SMS1- or SMS2-dependent NBD-PC formation. In contrast, addition of non-choline phospholipids (e.g. PI) had no effect. These data suggest that human SMS1 and SMS2, rather than functioning strictly as SM
20 synthases, are transferases capable of using PC and SM as phosphocholine donors to produce PC or SM, dependent on the relative concentrations of DAG and ceramide as phosphocholine acceptors, respectively.

Mammalian SM synthase was found to be sensitive to the bacterial PC-phospholipase C inhibitor, D609 (Luberto and Hannun, 1998).
25 In lysates of SMS1- or SMS2-expressing yeast cells, D609 inhibited SM synthesis in a dose-dependent manner. The extent of inhibition observed for SMS1-mediated SM synthesis (50% at 100 g/ml D609) was comparable to that reported for mammalian SM synthase (Luberto and Hannun, 1998). SMS2 proved two-fold less sensitive to the drug.

Hence, human SMS1, SMS2 and the mammalian SM synthase activity described in the literature share many enzymatic characteristics.

Example 6

Structure and expression of human SMS1 and SMS2

Figure 8A shows a sequence alignment of human SMS1, SMS2 and SMSr. Hydrophobicity analysis predicted six membrane-spanning alpha helices connected by hydrophilic regions that would form extramembrane loops. A comparative analysis with SMS and SMSr sequences from mice, *C. elegans*, *P. falciparum* and *Drosophila* revealed that the number and spacing properties of transmembrane helices are well conserved. In addition, SMS proteins contain four highly conserved sequence motifs, designated D1, D2, D3 and D4 (Figure 8A and B, residues highlighted in blue). Motifs D3 (C-G-D-X_3-S-G-H-T) and D4 (H-Y-T-X-D-V-X_3-Y-X_6-F-X_2-Y-H) are similar to the C2 and C3 motifs in LPPs (shared residues highlighted in red) and include the histidine and aspartate residues (underlined) that form a catalytic triad mediating the nucleophilic attack on the lipid phosphate ester bond Neuwald (1997).

As in LPPs, these residues are juxtaposed to transmembrane segments 4 and 6 of the SMS proteins and consequently would be oriented toward the same side of the membrane. This would suggest that motifs D3 and D4 are part of the catalytic site responsible for liberating cholinephosphate from PC during SM synthesis. Motifs D1 (P-L-P-D) and D2 (R-R-X_8-Y-X_2-R-X_6-T), on the other hand, appear entirely unique to SMS proteins and are located in the first extramembrane loop and third transmembrane helix, respectively (Figure 8A). The SMSr proteins found in humans, mouse, Drosophila and *C. elegans* each contain exact copies of the D1 and D3 motifs, yet exhibit one or more conserved

amino acid substitutions in motifs D2 and D4 (Figure 8A and B, residues highlighted in white on grey.

Previous work showed that some members of the mammalian LPP superfamily are expressed in only a limited set of tissues (Waggoner et al., 1999, Brauer et al, 1997).

Since differences in tissue distribution would provide a possible explanation for the existence of two different SM synthase isoforms in mammals, we investigated the expression profiles of human SMS1 and SMS2. As shown in Figure 10, Northern blot analysis detected the presence of a low abundant 3.8 kb transcript for SMS1 in human brain, heart, kidney, liver, muscle and stomach. A major 1.9 kb transcript for SMS2 was expressed to a similar level in all of the above human tissues. These results suggest that human SMS1 and SMS2 are encoded by ubiquitously expressed genes.

Example 7

Subcellular localisation and membrane topology

SM synthesis occurs in the Golgi complex as well as at the plasma membrane of mammalian cells. Endosomes have been put forward as another major site of SM synthesis (Kallen et al., 1994) but this has been disputed (van Helvoort et al., 1994).

It is not known whether SM synthase activity detected at these locations is due to the presence of more than one iso-enzyme in the cell. This led us to examine the subcellular distribution of V5-tagged versions of human SMS1 and SMS2 in transfected HeLa cells using immunofluorescence microscopy. As shown in Figure 11A, SMS1 was concentrated in the perinuclear region where it displayed extensive colocalisation with sialyltransferase, a marker of trans Golgi cisternae. This colocalisation was also observed in cells treated with nocodazole, a drug causing fragmentation of the Golgi by disrupting the microtubular network, hence confirming the association of SMS1 with the

Golgi apparatus. SMS2 displayed a different localisation pattern and was primarily concentrated at the plasma membrane (Figure 11B). A portion of SMS2 was also found in the perinuclear region where it colocalised with sialyltransferase (see Figure 12). This Golgi-associated pool of SMS2 unlikely represents newly synthesized material en route to the cell surface, since it was also observed in cells after a 4-hour chase with cycloheximide. There was no substantial colocalisation of SMS2 with markers of the endosomal/lysosomal system (e.g. EEA1, CD63, internalised transferrin; data not shown). Whether SMS2 cycles between the Golgi and the plasma membrane, and thereby passes through endosomes remains to be established.

Since SM synthesis takes place in the exoplasmic leaflet of the Golgi and the plasma membrane (Futerman et al., 1990; van Helvoort et al., 1994), the putative catalytic residues in motifs D3 and D4 of SMS1 and SMS2 would be oriented toward the Golgi lumen and cell surface, respectively, whereas their termini would be located on the opposite, cytosolic side of the membrane (Figure 13A). To test this prediction, we investigated the sidedness of the V5-tagged COOH termini of human SMS1 and SMS2 by protease protection analysis. When HeLa cells transfected with the SMS2-V5associated type I membrane protein, p24 construct were trypsinised, the V5 tag remained intact, unless cells were lysed or treated with detergent prior to incubation with the protease (Figure 13B). Trypsinisation of lysates from SMS1-V5 expressing cells in the absence of detergent resulted in a complete removal of the V5 tag. Under these conditions, the Golgi-associated type I membrane protein, p24 (Gommel et al., 1999).

was largely protected. Collectively, these results indicate that the COOH termini of SMS1 and SMS2 are cytosolic. Consequently, the putative active site residues in these proteins would be positioned on the exoplasmic leaflet (Figure 13A), hence where SM synthesis was found to occur. Together, these findings demonstrate yet another level of similarity between SMS1, SMS2 and the SM synthase activity previously described in mammalian cells.

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FIG. 9

Sequence listings

Sequence listings 1 - 11: conserved sequence motifs of phosphotransferases as indicated in figure 8 B;

sequence listing 1: partial polypeptide sequences of *hu* SMS1
sequence listing 2: partial polypeptide sequences of *hu* SMS2
sequence listing 3: partial polypeptide sequences of *ce* SMS1
sequence listing 4: partial polypeptide sequences of *ce* SMS2
sequence listing 5: partial polypeptide sequences of *ce* SMS3
sequence listing 6: partial polypeptide sequences of *pf* SMS1
sequence listing 7: partial polypeptide sequences of *pf* SMS2
sequence listing 8: partial polypeptide sequences of *hu* SMSr
sequence listing 9: partial polypeptide sequences of *dm* SMSr
sequence listing 10: partial polypeptide sequences of *ce* SMSr
sequence listing 11: partial polypeptide sequences of *ce* SMSdr

Sequence listing 12: *hu*SMS1

MKEVVYWSPKKVADWLLENAMPEYCEPLEHFTGQDLINLTQEDFKKPPLCRVSSDN
GQRLLDMIETLKMEHHLEAHKNGHANGHLNIGVDIPTPDGSFSIKIKPNGMPNGYRK
EMIKIPMPELERSQYPMEWGKTFLAFLYALSCFVLTTVMISVVHERVPPKEVQPPLPD
TFFDHFNRVQWAFSICEINGMILVGLWLIQWLLLKYKSIISRRFFCIVGTLYLYRCITM
YVTTLPVPGMHFNCSPKLFGDWEAQLRRIMKLIAGGGLSITGSHNMCGDYLYSGHTV
MLTLTYLFIKEYSPRRLWWYHWICWLLSVVGIFCILLAHDHYTVDVVVAYYITTRLF
WWYHTMANQQVLKEASQMNLLARVWWYRPFQYFEKNVQGIVPRSYHWPFPWPV
VHLSRQVKYSRLVNDT

Fig 9 (Cont.)

Sequence listing 13: *hu*SMS2

MDIIETAKLEEHLENQPSDPTNTYARPAEPVEEENKNGNGKPKSLSSGLRKGTKKYPD
YIQIAMPTESRNKFPLEWWKTGIAFIYAVFNLVLTTVMITVVHERVPPKELSPPLPDKF
FDYIDRVKWAFSVSEINGIILVGLWITQWLFLRYKSIVGRRFCFIIGTLYLYRCITMYVT
TLPVPGMHFQCAPKLNGDSQAKVQRILRLISGGGLSITGSHILCGDFLFSGHTVTLTLT
YLFIKEYSPRHFWWYHLICWLLSAAGIICILVAHEHYTIDVIIAYYITTRLFWWYHSMA
NEKNLKVSSQTNFLSRAWWFPIFYFFEKNVQGSIPCCFSWPLSWPPGCFKSSCKKYSR
VQKIGEDNEKST

Sequence listing 14: *ce*SMS1

MKMSWNHQYTNYGSIADDNGDEEKAENSEGAAAEKVEKQHDDDGVVVHEETDGV
ASSRSSHHDKQKPGETKKSGDGKMDDDDIITTARSSSRRICGSAASSSDSETADDAPL
LPDEGPSHAVRLEMPGDKPASPHDRFPKTPLKTLVAFLMLVVAAAGNTITLSWIHER
YPLTPPLPDIVFELIPKIPWGLRLCENLMIGSFVSLLVLILFHRHRWIVLRRLCFIGSILYG
MRCITMMVTPVPKADEDFECSPRFGENATFSLIVMRGVWSMFGLGLNLFDNQKVVL
CGDYIYSGHTLVLVVSALFIGEYSPRRFYILHWLSWLVCSVGVIFLVLSHGHYTIDVIL
SYFACTRVFWAYHTQAAHPSIRLSVQNHQAKEFWFPLLRWFEGDIRRPVPRRFDCPIS
YSQTPVDYSFLPWFSFFFQSNPMMFPGAFTFYHTQNHSKSTMSLPK

Sequence listing 15: *ce*SMS2

MTNSSEFTDVLQSRDPCVSNGIVINIDPIDPEPTPIRKEFTCEDTFHHEHHGNSEGFKTL
TAFLCLMLSAFLNFFLLTVIHDVVPRQPLPDLTFMIIPQQRWAWSVGDVLSTVSSVVA
FTIIFLHHQRWIVLRRTFLLGAIMYGLRAVILGVTFLPPSFHNRDEICQPQVNRTAMYG
MEIATRFLTYVITLGLTSGQDKILCGDLMFSGHTVVLTIMYFVQLQYTPRGLVILRYIA
APITFLGIAALVVSGGHYTMDVLIAYWLTSHVFWSYHQIFEMRKDDRPQAPLSRLW
WFWLCYWFESDVADGKLVNKWNWPLEGPQRMHTIMNRINYKLQ

Fig 9. (Cont.)

Sequence listing 16: *ce*SMS3

MGSVSKTVISARGASPDDEQNGTKNGISNGSEWAKCIFLFFFLFIAGMSNWAVLAYT
HDYVPRESLPDIVFSLVSEQRWASSLGDFCVALCIVMLGALLVIHQHRGTILKRVVFC
AGTLYAMRSVTLAATQLPSGYTDNQGRCRDQVESEAGVFFGRLFEQTIRIGFQSKDQ
MLCGDLLFSGHTLVMVTCSLAVAYYLPKSIKPLQWVSHVACLIGMICMTISRTHYTID
VVIAYWLSNMVFRMYHAYCEVDMCMERRKSILYSWWPCRIVDWLEQDIVPGRLEN
RCQLPWRRSTPRGQERGGASAESSDSSVTMCDNITTSHHQKHVSISSSSTYPLPC

Sequence listing 17: *pf*SMS1

MIEGNINANTTHQNTTNNKIEKNFIKEKDIINNDTIKILIEHNNSFNDESTNYITTFNDIK
DKHYNHKDSYSDNKGKIKTLRSNFSDIQDEVIITNIGHYFEGQENIKKSVEIKNCPYKK
KMLKIFLRRLVCATIIGIIGICIQCYFIILSDTYYKTGDEPLKDRLHEIFKEIPAFMNTPFV
NGSIMFFLAITLLRFGLFCPLLLSITILIRIILMLSFIYCIRSFFIYVTTLPCPIPTCQPLKHKT
LVENLYTFYLIITAQVYECTDLVISGHTAFTTLLTFFWFFYERNIYVKTTIFLYSIYIYIIIII
SRFHYTVDVLMGYVFGGSVFLFYHYLVDVAARRYALNTSVFPQTYGFSGSFTDRFQ
VFQYFIRAITYLEALDHRMNFALSYDKEWNCFCSCVPVNKNSLLIKKKNVRNEEYYD
FSDHFYHSYAGNGTYNLSTIRNIIKEFKRFFGMNKKN

Sequence listing 18: *pf*SMS2

MNKYEYFYSPIQLLHLILLLRPILYENYTYDENDKSSIGTDMYEINMNRKMSNISISRN
STINEEEILSEYRLCKILLIKLMFALLFLLIALIIQGFFMIYSDSYYKSNTQPLSDRIHDLF
GNPPKWISYKLSNTLIAILTLSFLKIILFNSIYLSIAIICRFLYIVGSFYIIRGLLIYVTSLPAT
LETCLPLESGNFLFNLLQIIKINTNLVYYVCADLIVSGHSFSTTIFLMFSFYYINNVIIKFIIF
TFSCFIYAIIIIGFIHYTSDVLLGIIFGVFMFSFYHIMLDISSQYYIFNKLFEIKIISNNKNIHA
KPFFLRFFVARIFFKIIPYLEGLNYTLDYAINKNNDLSTFCNCDHDNNKIPLFSFYKPITE
DKIIINYSDHLYHSYAGDGTINFLFWKFLKTIKKGLHK

Fig 9. (Cont.)

Sequence listing 19: *hu*SMSr

MAGPNQLCIRRWTTKHVAVWLKDEGFFEYVDILCNKHRLDGITLLTLTEYDLRSPPL
EIKVLGDIKRLMLSVRKLQKIHIDVLEEMGYNSDSPMGSMTPFISALQSTDWLCNGEL
SHDCDGPITDLNSDQYQYMNGKNKHSVRRLDPEYWKTILSCIYVFIVFGFTSFIMVIV
HERVPDMQTYPPLPDIFLDSVPRIPWAFAMTEVCGMILCYIWLLVLLLHKHRSILLRRL
CSLMGTVFLLRCFTMFVTSLSVPGQHLQCTGKIYGSVWEKLHRAFAIWSGFGMTLTG
VHTCGDYMFSGHTVVLTMLNFFVTEYTPRSWNFLHTLSWVLNLFGIFFILAAHEHYSI
DVFIAFYITTRLFLYYHTLANTRAYQQSRRARIWFPMFSFFECNVNGTVPNEYCWPFS
KPAIMKRLIG

Sequence listing 20: *dm*SMSr

MCDGEIGDPVTQPRSEGGGLVTMDQETRTHYLDAATDKHLTNGSPDPEPVDPLLVA
QWSIENVTSWATCMEHFSRTLLDCLRQEAIDGEVLLSLTEEDVRDMRYKLGYKLTFG
ELKKFWIAVLKLQLLVKNSSAESVILGIECHGNGNSVYMPLASTGCGPPSSSTCPCPQ
AECPSYVSDCDTYLRMGGRYVPPEYFKTAMSLGYSFVVTWITSLTMVIVHERVPDM
KRYPPLPDIFLDNVPHIPWAFNMCEITGSLLFTIWVVVLTFHKYRLVLLRRFFALAGTV
FLLRCVTMLITSLSVPGTHLQCSQKDFAIDDPNVDMVGALIIRMSRAYRIWSGLGMSI
QGVRTCGDYMFSGHTVALTLLNFFITEYTPRNLYFLHTLTWLLNMFGIFFILAAHEHY
SIDVFVAFYITSRLFLYYHTLANNRALMQSDSKRTRVWFPMFSYFESSVDGMVPNEY
DTLGSLIDGIIEQIFKAKDQLAISVKRCWLDAPLSGNSSAHMFGGESEQCLRNGTASA
AFFSPHQSLIGGLGGQRSQTHLNSAQSGSSTPTPATSAPTKSLPTQKKTFRDASVDPFS
RTTFAAVQQAENVKDSPMKEKKHL

Sequence listing 21: *ce*SMSr

MLDNRPIPADPNEWRCEDVGNWLKKIGMAKYADLIAMKHKVDGKCLLALTDTDLK
DPPVSINCLGDIKKILFAIEFLSQKVVEIGNSGVHHRSTPNGNGPSLKNSKDGLLVEYN
EQNHLSISGEDVYTTTRRAEIVEDEETLLDTLAKSSDGTSTVQLISREEIIRQVERPDTY
FKSVAKLLIAFAYSSLSFLMTSFVMVLVHDRVPDTKTYPPLPDIVLDNVPHIPWAFDM

Fig 9 (Cont.)

CETIGLVLAVVWFTVLFFHNQRVIVARRMFSLLGTVFLLRCFTMLITSLSVPGIHLQCE
ARPNTTMQEKLHKAFHIWSNLGMSLHGVRSCGDYMFSGHTTVITMISHFITEYTPAD
WTGLHTFTWVLNCFAIFLILAAHEHYSIDVFIAFYISSRMFLYYHAYAYNHAGITATD
YRMRTWFPLGWFFEYGSQGKVENEFSLPINIRIPRRVFFAKSEEPKITPKSDSSRKRSS
VVAAKQNGNSKNHTKKHN

Sequence listing 22: *ce*SMSdr

MCKSCENYGKFFFAMLKICIDNSSNFAMKKDAENENLLHAYEHDEQSSKYFIKPDSR
YSIDDDISVKIDGKQLEVRKWPTLLATAMVGVGWLSNEVALAWVHERVPDDYHPLP
DLFFSHFPEVKERLKIFGILGKFEKSEKKTSEFSSPIRGAIRIAEYIMMILLISALLVMFTH
QHRWIVIRRVFFCIAMAYSFRALCVTIFQVPVPSINTYCAPKSNSSLELVAGRVVKMF
WSAGIEQLRPRELCGDLIVSGHTLTIFTAFLVFKTYAPQRLQPLSHIYHVLAFTALFSIL
LARKHYMIDIVLGYTVSTRIFMEYHALAASYHNRTFETNPLAWSFWSFFIPIFECDAPA
NMHNHLLLYNRSTSSKNVSTLKKSRRSFE

Claims

1. An isolated polypeptide comprising one or more of the amino acid motifs selected from the group consisting of a sequence with at least 80% identity to any of

(a) P-L-X-D-X(35,75)-R-R-X(8)-[YF]-X(2)-R-X(6)-T

(b) C-X-D-X(3)-S-G-H-T

(c) H-Y-[TS]-X-D-[VI]-X(3)-[FYI]-X(6)-F-X(2)-Y-H.

2. A polypeptide according to claim 1 that is derived from the group consisting of *Animalia, Alveolata* and *Kinetoplastida.*

3. A polypeptide according to claim 1 or 2 selected from the group comprising any of the SEQ ID No. 1 - 11 or at least 70% similarity thereto.

4. A polypeptide according to claim 1 or 2 comprising an amino acid sequence
with at least 70 % similarity to any of the SEQ ID No. 12 - 22.

5. A polypeptide according to any one of the previous claims comprising an amino acid sequence with at least 20 % identity to any of the SEQ ID No. 12 - 22.

6. A polypeptide according to claim 5 comprising an amino acid sequence with at least 30 % identity to SEQ ID No. 12.

7. A polypeptide according to claim 5 comprising an amino acid sequence with at least 40 % identity to any of the SEQ ID No. 19 - 21.

8. A polypeptide according to any one of the claims 1 - 3 comprising an amino acid sequence with at least 22 % identity to SEQ ID No. 22.

9. A polypeptide according to any one of the claims 1 - 6 with sphingomyelin synthase activity.

10. A polypeptide according to any one of the claims 1 - 5 and 7 with ethanolamine phosphorylceramide synthase activity.

11. A polypeptide according to any one of the claims 1 - 5 and 8 with one or more of the activities selected from the group consisting of phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase.

12. A nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences as described in claim 9 and an anti sense nucleotide sequence that is complementary thereto.

13. A nucleotide sequence selected from the group consisting of a nucleotide sequence coding for any of the amino acid sequences as described in claim 10 and an anti sense nucleotide sequence that is complementary thereto.

14. A nucleotide sequence selected from the group consisting of a nucleotide

sequence coding for any of the amino acid sequences as described in claim 11 and an anti sense nucleotide sequence that is complementary thereto.

15. A plasmid comprising any of the nucleotide sequences described in any of the
claims 12, 13 or 14.

16. A vector comprising any of the nucleotide sequences described in any of the claims 12, 13 or 14.

17. A (micro)organism or cell line in which any of the nucleotide sequences described in claim 12 was introduced.

18. A (micro)organism or cell line in which any of the nucleotide sequences described in claim 13 was introduced.

19. A (micro)organism or cell line in which any of the nucleotide sequences described in claim 14 was introduced.

20. A process for producing sphingomyelin synthase comprising the expression of
any one of the nucleotide sequences described in claim 12 in a (micro)organism or cell line of claim 17 and the isolation of sphingomyelin synthase.

21. A process for producing sphingomyelin comprising the expression of the nucleotide sequences described in claim 12 in a (micro)organism or cell of claim 17 and the isolation of sphingomyelin.

22. Use of one of more of the nucleotide sequences of claim 12 to influence the reaction

$$CER + PC \leftrightarrow SM + DAG$$

in vivo or *in vitro*.

23. Use of one of more of the nucleotide sequences of claim 12 to identify or develop compounds influencing the reaction

$$CER + PC \leftrightarrow SM + DAG$$

in vivo or in vitro.

24. A process for producing ethanolamine phosphorylceramide synthase comprising
the expression of any one of the nucleotide sequences described in claim 13 in a (micro)organism or cell line of claim 18 and the isolation of ethanolamine phosphorylceramide synthase.

25. A process for producing ethanolamine phosphorylceramide comprising the
expression of the nucleotide sequences described in claim 13 in a (micro)organism or cell line of claim 18 and the isolation of ethanolamine phosphorylceramide.

26. Use of any one of the nucleotide sequences of claim 13 to influence the reaction

$$CER + PE \leftrightarrow EPC + DAG$$

in vivo or *in vitro*.

27. Use of any one of the nucleotide sequences of claim 13 to identify or develop compounds influencing the reaction

$$CER + PE \leftrightarrow EPC + DAG$$

in vivo or *in vitro*.

28. The application of the compounds of claim 23 or 27 in medical use.

29. The application of the compounds of claim 28 for the manufacture of medicaments treating a disease selected from the group consisting of cancer, metabolic diseases and diseases caused by parasites.

30. A process for producing phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase comprising the expression of any one of the corresponding nucleotide sequences described in claim 14 in a (micro)organism or a cell line of claim 19 and the isolation of phosphatidyl:glycoprotein cholinephosphotransferase or phosphatidyl:glycolipid cholinephosphotransferase.

31. A process for producing phosphorylcholine-substituted glycoprotein or

phosphorylcholine-substituted glycolipid comprising the expression of the corresponding nucleotide sequences described in claim 14 in a (micro)organism or cell of claim 19 and the isolation of phosphorylcholine-substituted glycoprotein or phosphorylcholine-substituted glycolipid .

32. Use of one of more of the nucleotide sequences of claim 14 to influence the reaction

glyco lipid/protein + PC ↔ PC-substituted glyco lipid/protein + DAG

in vivo or *in vitro*.

33. Use of one of more of the nucleotide sequences of claim 14 to identify or develop compounds influencing the reaction

glyco lipid/protein + PC ↔ PC-substituted glyco lipid/protein + DAG

in vivo or *in vitro*.

34. The application of the compounds of claim 33 in medical use.

35. The application of the compounds of claim 34 for the manufacture of a medicament treating a disease caused by parasitic nematodes.

36. A process to isolate candidates for functional genes of a previously unidentified enzyme with known activity from a huge database by combining at least four characteristics based on data from bio-informatics and from biochemistry, viz.

– presence of a sequence motif shared with previously identified enzymes having a related function
– biochemical function of the gene should be unknown until now
– no structural homologues in an organism that does not contain the enzyme
– ability to mediate a reaction catalysed by the unidentified enzyme upon its heterologous expression in an organism or cell lacking said enzyme activity.

37. A process to isolate candidates for functional genes according to claim 36 characterized by also considering the presence or non-presence of transmembrane domains depending on the working mechanism of the enzyme in relation to the membrane.

38. A method for determining whether a compound is capable of modulating an enzymatic activity displayed by a cell, said activity comprising an activity of an enzyme of the group of enzymes identified as sphingomyelin synthases, ethanolamine phosphorylceramide synthases, phosphatidylcholine:glycoprotein cholinephosphotransferase and phosphatidylcholine:glycolipid cholinephosphotransferase, said method comprising providing said cell with a nucleic acid encoding a polypeptide according to any one of claims 1-11, contacting said cell with said compound and determining whether said enzymatic activity is modulated.

39. A method according to claim 38 wherein said cell is deficient in sphingomyelin synthase activity.

40. A method according to claim 38 or claim 39, wherein said cell is a cell of a eukaryotic micro-organism.

41. A method according to claim 40, wherein said cell is yeast cell.

42. A method according to any one of claims 38-41, wherein said polypeptide comprises a sequence as depicted in figure 8 or a functional part, derivative and/or homologue thereof.

43. A method according to claim 42, wherein said polypeptide comprises a sequence as depicted in figure 8A or a functional part, derivative and/or homologue thereof.

44. A method according to any one of claims 38-43, wherein said polypeptide is derived from a plasmodium.

45. A method according to claim 44, wherein said plasmodium sequence is a sequence as depicted in figure 8B or a functional part, derivative and/or homologue thereof.

46. A method according to any one of claims 38-45, wherein said compound comprises RNA.

47. Use of a nucleic acid encoding a polypeptide according to any one of claims 1-11, as a probe.

48. Use of an oligonucleotide specific for a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof, for detecting said sequence.

49. Use according to claim 47 or claim 48, for assessing whether a cell comprises sphingomyelin synthase activity.

50. Use of an inhibitor of a sphingomyelin synthase according to any one of claims 1-11, as a cell death promoter.

51. Use according to claim 50, wherein said cell is a cell of a parasite.

52. Use according to claim 50, wherein said cell is a human cell, preferably a tumor cell.

53. Use of a nucleic acid according to any one of claims 12-14, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof for enhancing cell survival and/or cell growth.

54. A method for at least in part improving the yield of an secretion product of a cell comprising providing said cell with a polypeptide according to any one of claims 1-11, or a nucleic acid according to any one of claims 12-14, preferably comprising a nucleic acid sequence encoding a polypeptide as depicted in figure 8 or a functional part, derivative and/or homologue thereof.

55. A method according to claim 54, wherein said cell is a cell of a eukaryotic micro-organism.

56. A method according to any one of claims 38-46, further comprising providing said cell or a fraction thereof with a labelled substrate for said sphingomyelin synthase.

57. A method according to claim 56, further comprising harvesting sphingolipid from said cell or said fraction and detecting labelled sphingolipid.

58. A method according to claim 56 or 57, further comprising detecting said labelled sphingolipid using (thin layer) chromatography or mass spectrometry.

59. A method for targeting a first polypeptide according to any one of claims 1-11 to a different cellular compartment comprising providing a cytosolic part of said first polypeptide with a cellular compartment localization signal of a cytosolic part of a second polypeptide according to any one of claims 1-11, wherein said first and said second polypeptide, when unmodified, reside in different cellular compartments.

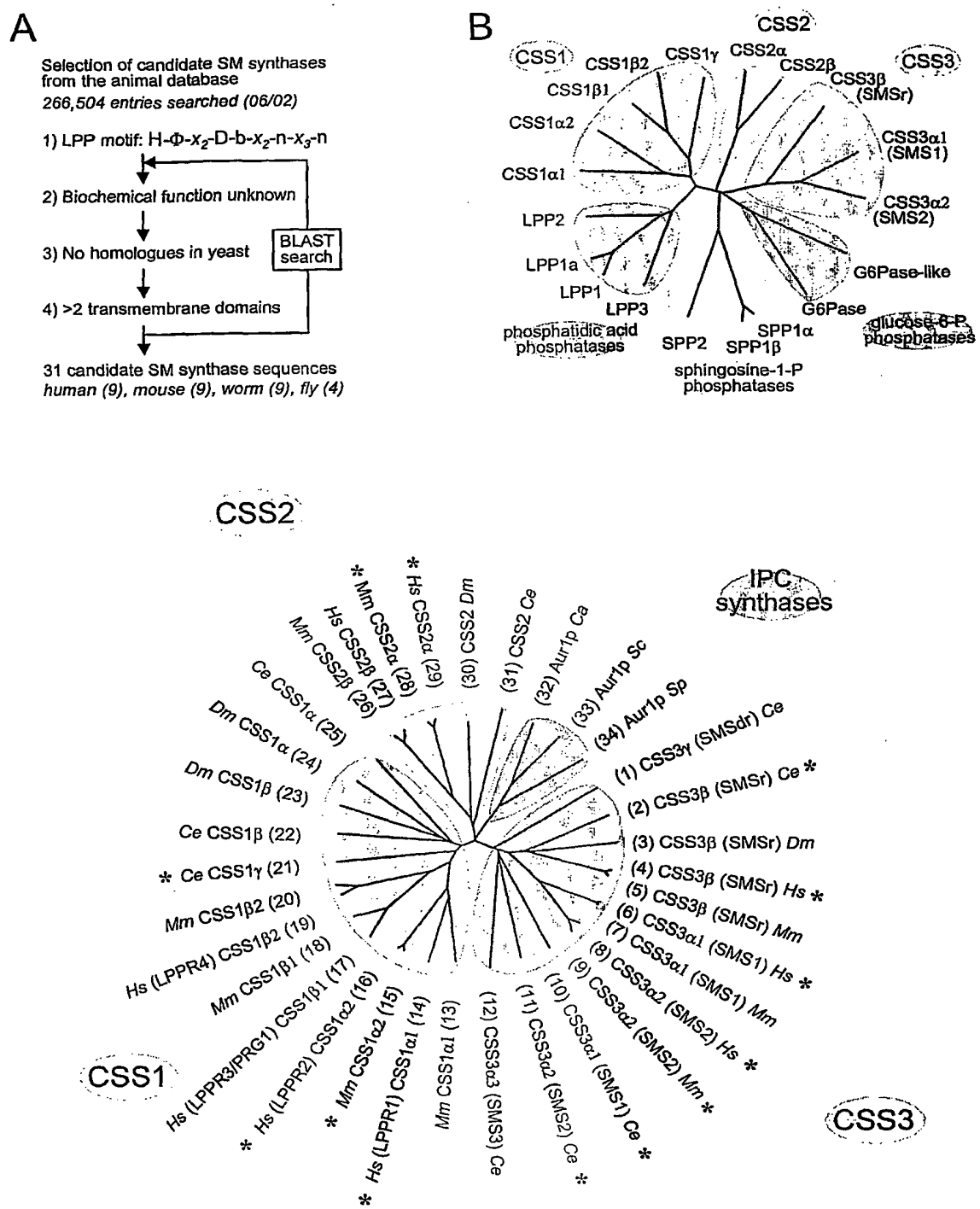
60. A method according to claim 59, wherein said cytosolic part of said first polypeptide comprises the C-terminal end of said polypeptide.

61. A method according to claim 59 or claim 60, wherein said cytosolic part of said second polypeptide comprises the C-terminal end of said polypeptide.

62. A method according to any one of claims 59-61, wherein said cellular compartments comprises the plasma membrane, the endosomal compartment, the Golgi, the endoplasmatic reticulum or a combination thereof.

63. A method according to any one of claims 59-62, wherein said cellular compartment localization signal of said second polypeptide replace the C-terminal cytosolic part of said first polypeptide.

Fig. 1


A
Selection of candidate SM synthases from the animal database
266,504 entries searched (06/02)
1) LPP motif: H-Φ-x2-D-b-x2-n-x3-n
2) Biochemical function unknown
3) No homologues in yeast
BLAST search
4) >2 transmembrane domains
31 candidate SM synthase sequences
human (9), mouse (9), worm (9), fly (4)
B
CSS2
CSS1
CSS3
CSS1β2
CSS1γ
CSS2α
CSS2β
CSS3β (SMSr)
CSS1β1
CSS1α2
CSS3α1 (SMS1)
CSS1α1
CSS3α2 (SMS2)
LPP2
LPP1a
G6Pase-like
LPP1
LPP3
G6Pase
phosphatidic acid phosphatases
SPP2
SPP1β
SPP1α
glucose-6-P phosphatases
sphingosine-1-P phosphatases
CSS2
IPC synthases
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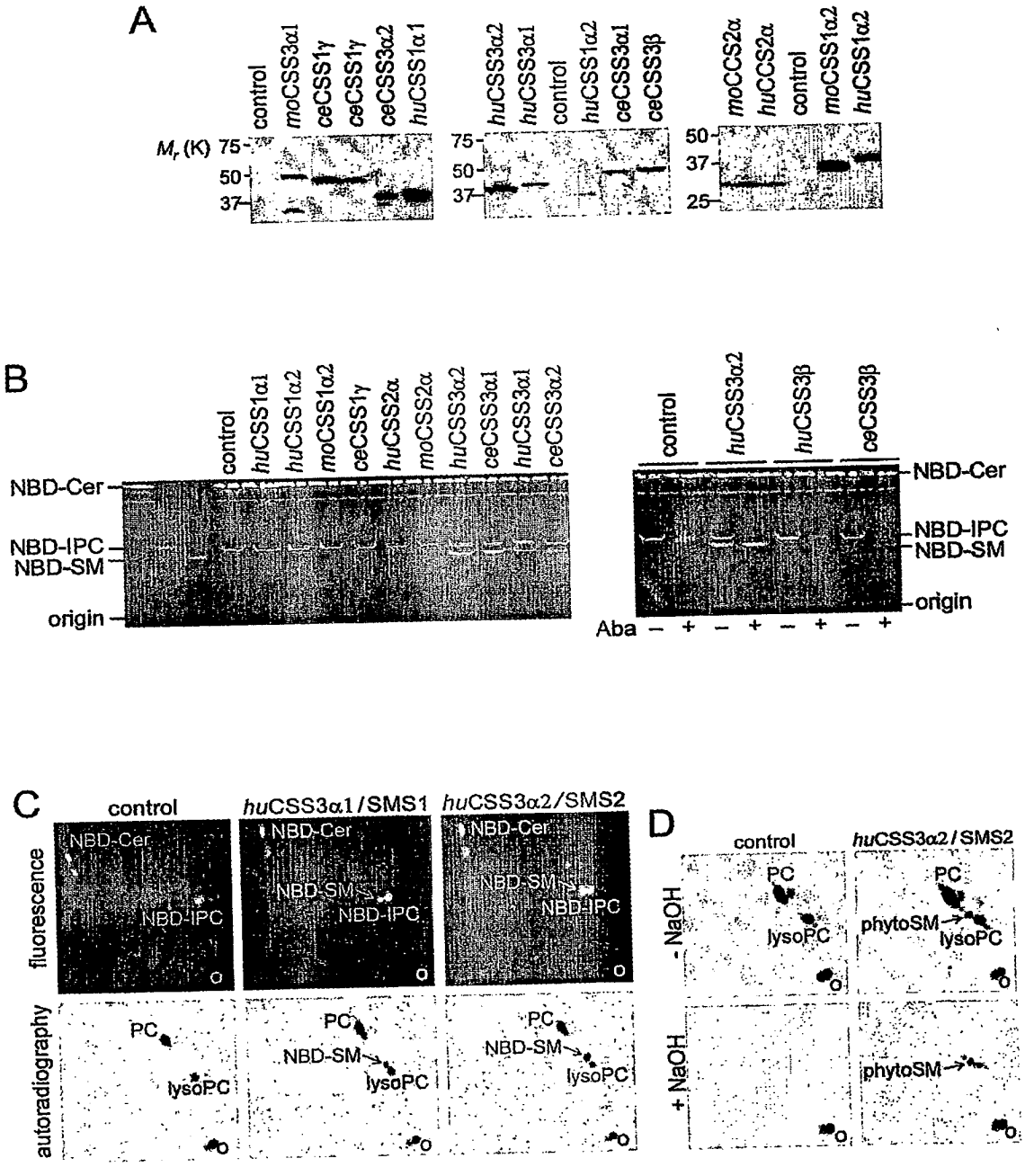
CSS1
CSS3

Fig. 2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37
75
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B
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NBD-IPC
NBD-SM
origin
Aba – + – + – + – +
C
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NBD-SM
autoradiography
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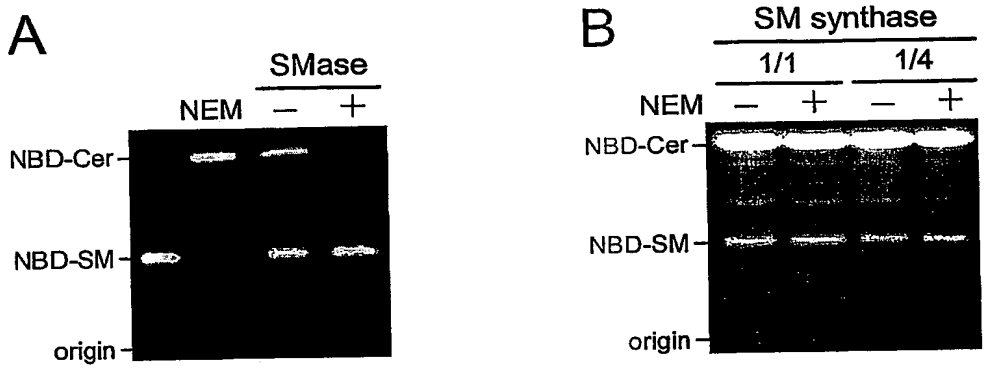
Fig. 3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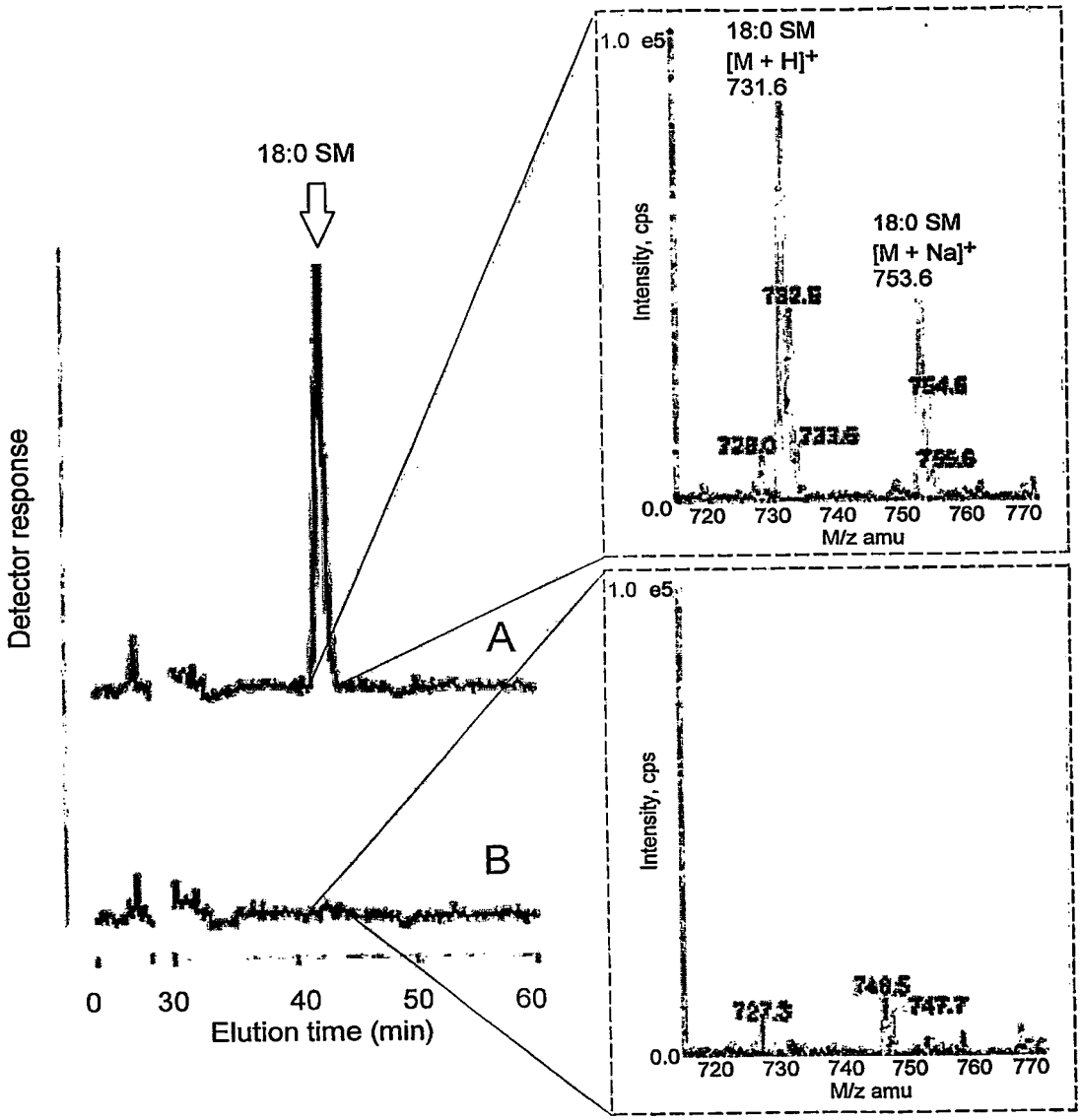
origin

Fig. 4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Intensity, cps
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733.5
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754.6
755.6
0.0
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730
740
750
760
770
M/z amu
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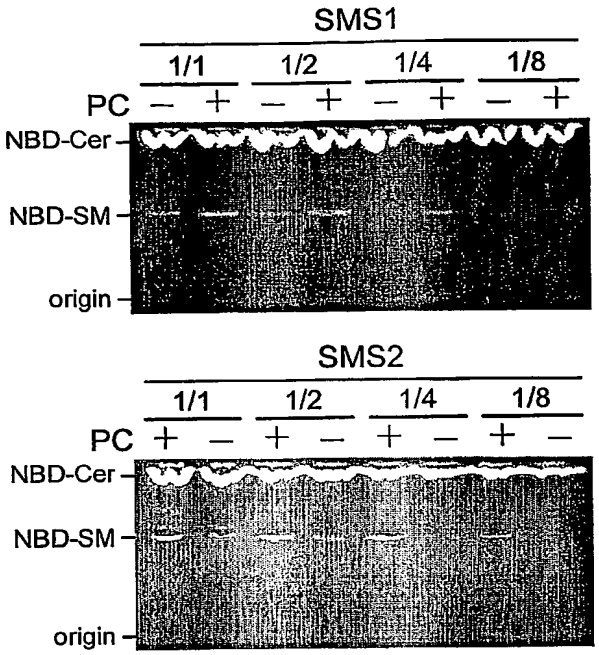
770
M/z amu

Fig. 5


SMS1
1/1
1/2
1/4
1/8
PC
– + – + – + – +
NBD-Cer
NBD-SM
origin
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+ – + – + – + –
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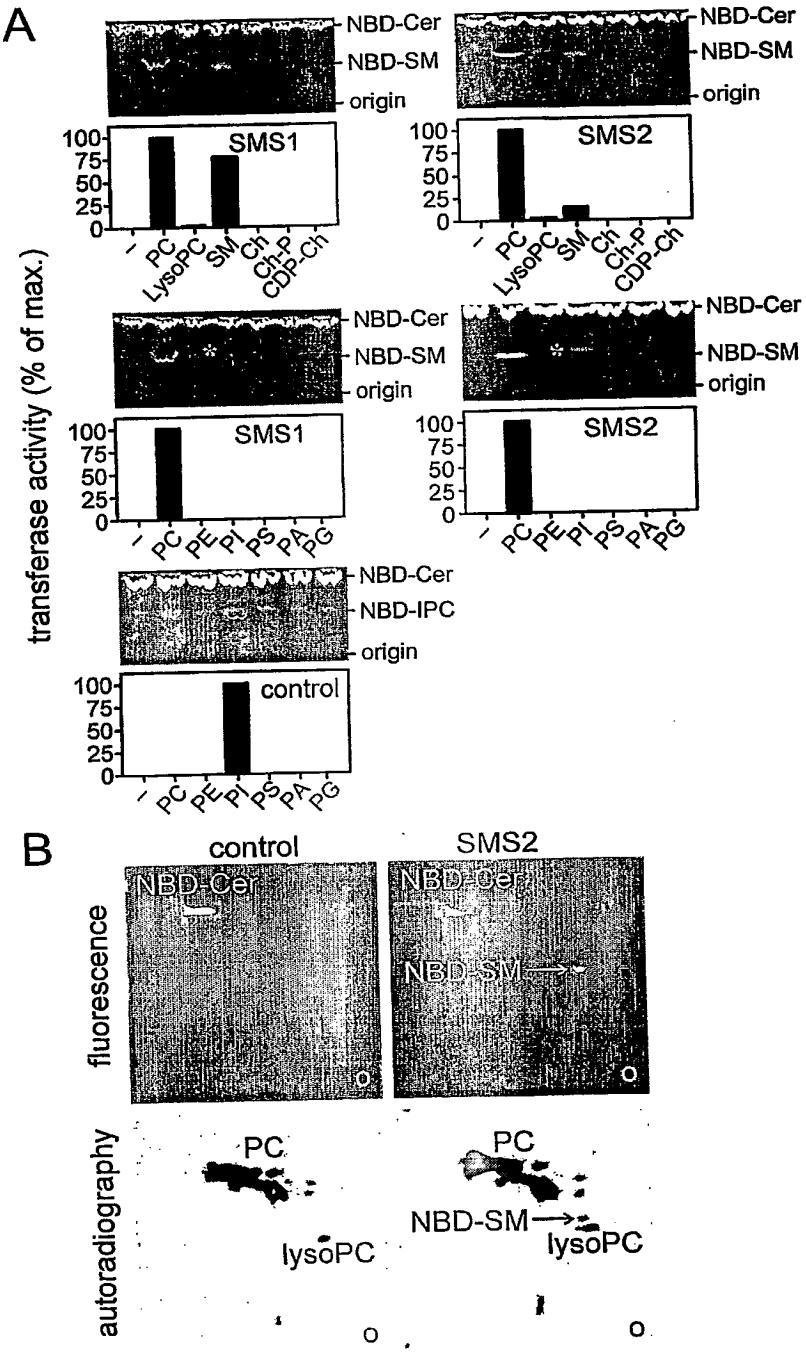
NBD-SM
origin

Fig. 6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75
50
25
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LysoPC
SM
Ch
Ch-P
CDP-Ch
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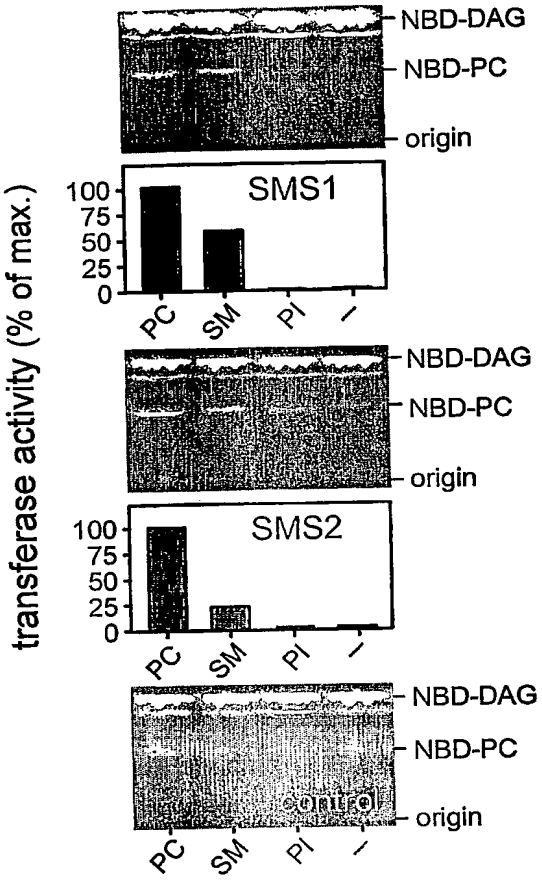
Fig. 7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100
75
50
25
0
PC
SM
PI
–
transferase activity (% of max.)
NBD-DAG
NBD-PC
origin
SMS2
100
75
50
25
0
PC
SM
PI
–
NBD-DAG
NBD-PC
origin
PC
SM
PI
–

Fig. 8

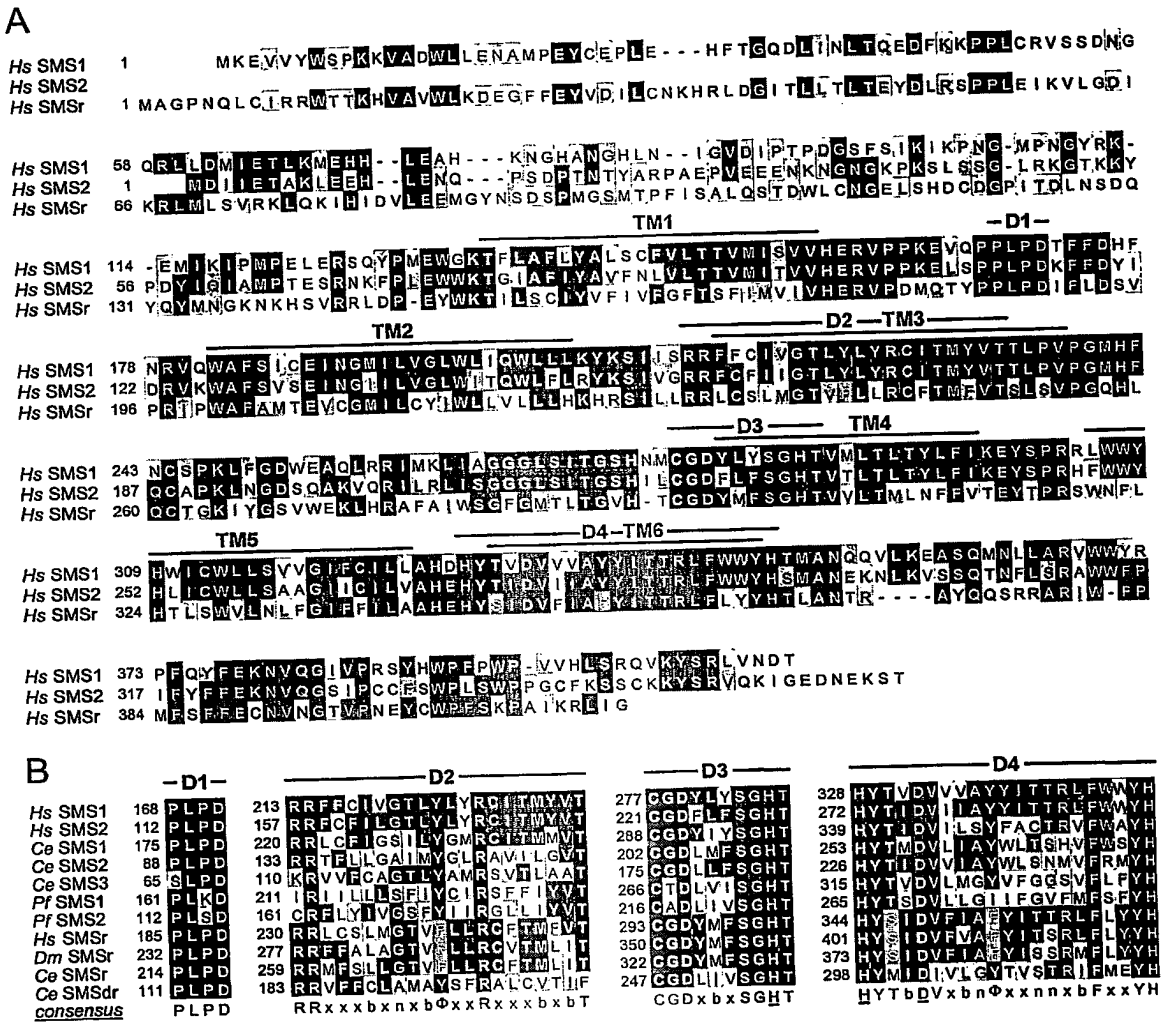

A
Hs SMS1
Hs SMS2
Hs SMSr
TM1
D1
TM2
D2
TM3
D3
TM4
TM5
D4–TM6
B
D1
D2
D3
D4
Hs SMS1
Hs SMS2
Ce SMS1
Ce SMS2
Ce SMS3
Pf SMS1
Pf SMS2
Hs SMSr
Dm SMSr
Ce SMSr
Ce SMSdr
consensus
PLPD
RRxxxbxnxbΦxxRxxxbxbT
CGDxbxSGHT
HYTbDVxbnΦxxnnxbFxxYH

Fig. 10

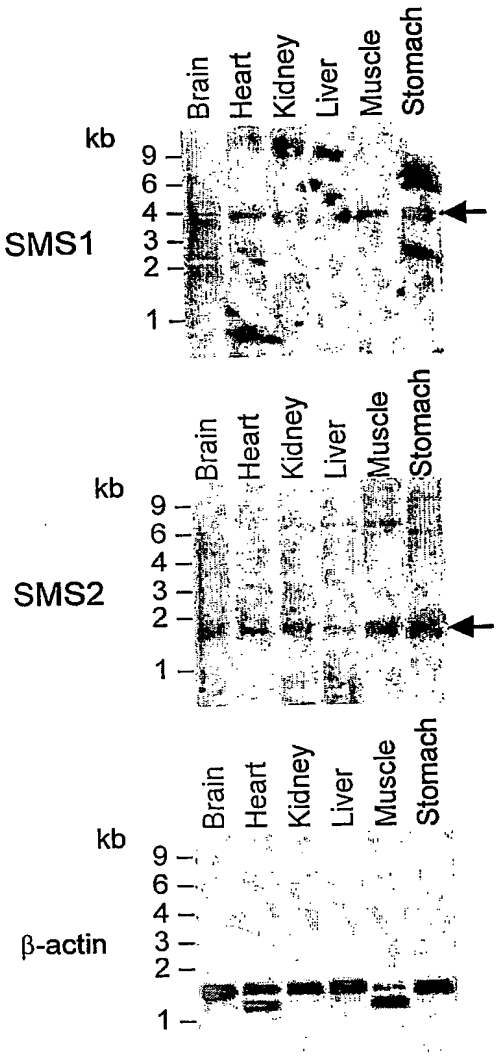

Brain
Heart
Kidney
Liver
Muscle
Stomach
kb
9
6
4
3
2
1
SMS1
SMS2
β-actin

Fig. 11

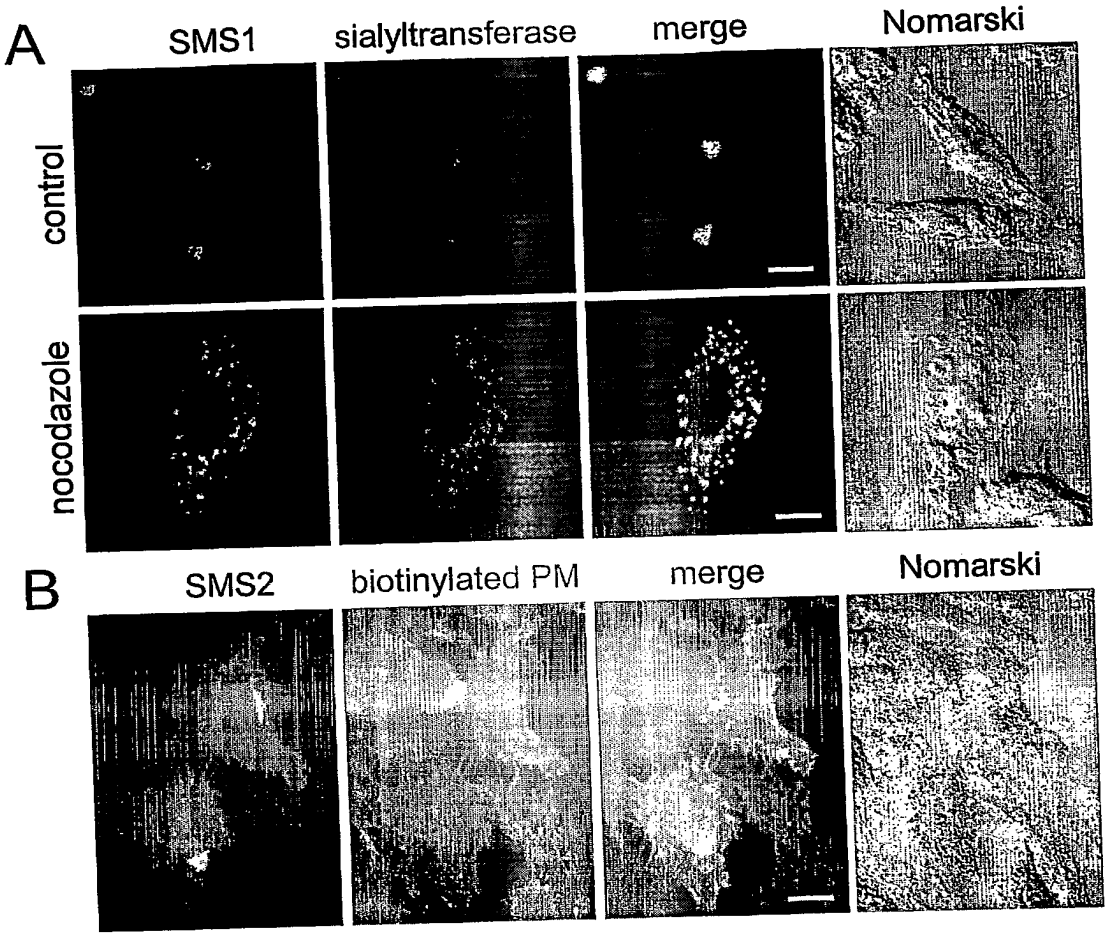

A
SMS1
sialyltransferase
merge
Nomarski
control
nocodazole
B
SMS2
biotinylated PM
merge
Nomarski

Fig. 12

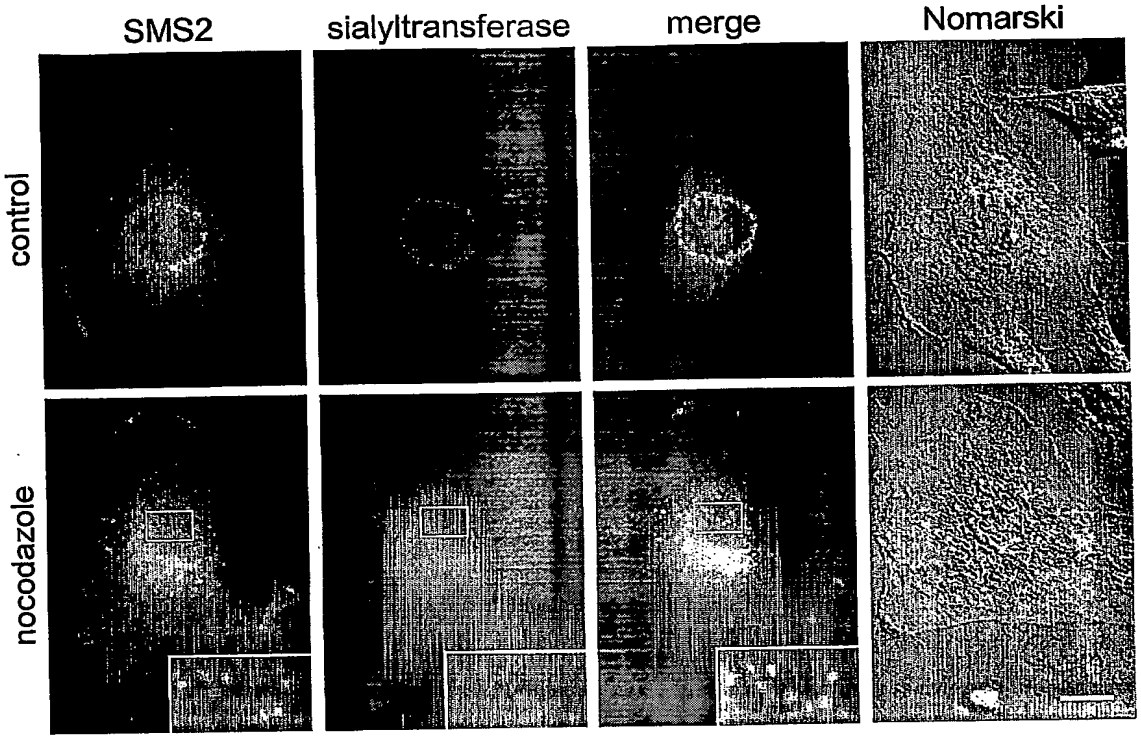

SMS2
sialyltransferase
merge
Nomarski
control
nocodazole

Fig. 13

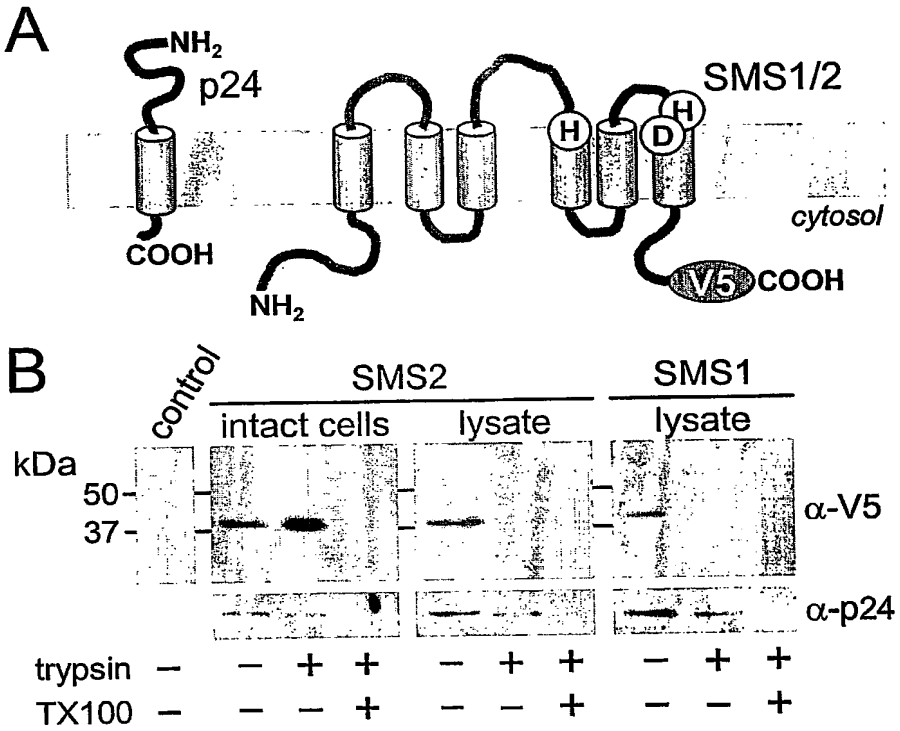
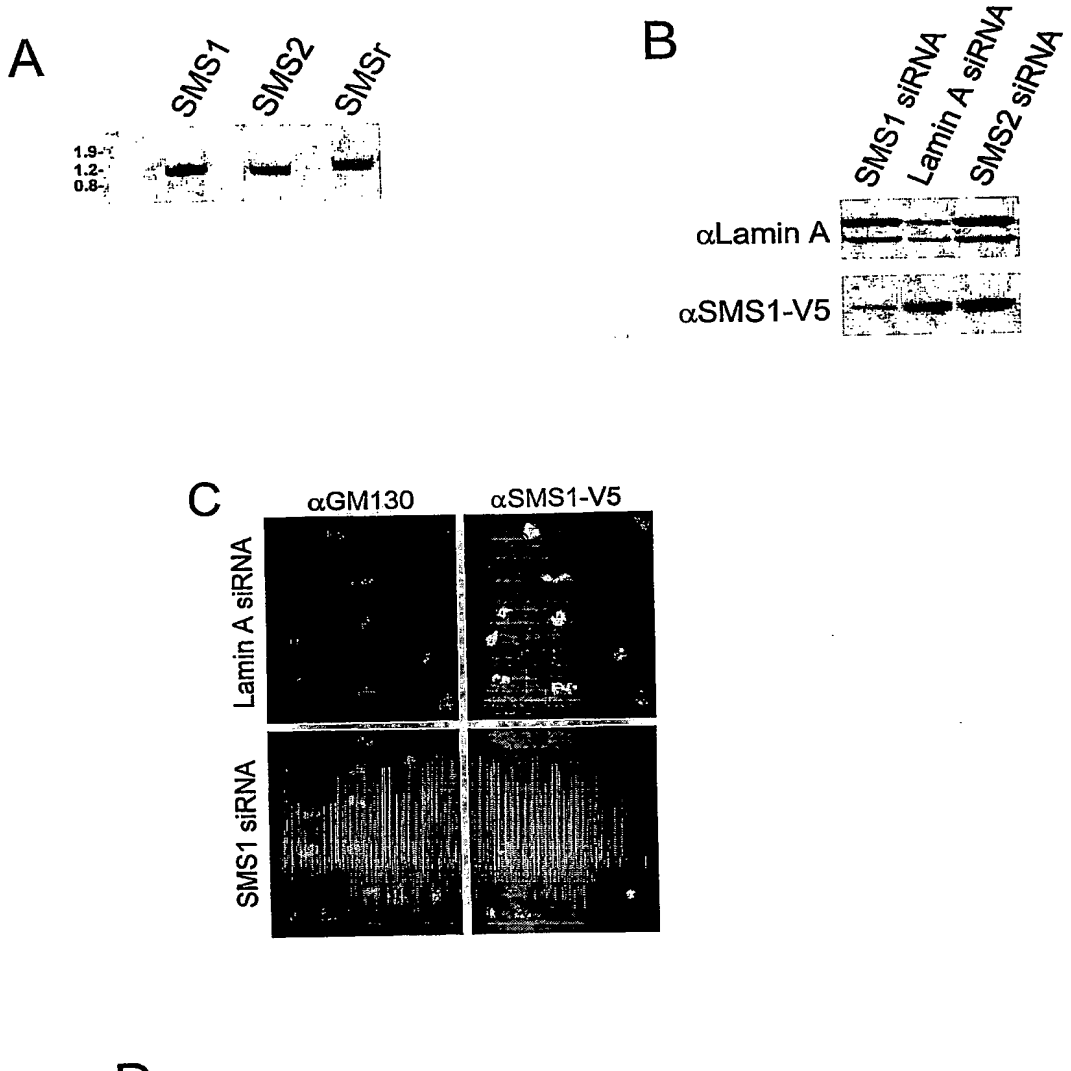

A
NH2
p24
SMS1/2
H
D
H
cytosol
COOH
NH2
V5
COOH
B
control
SMS2
SMS1
intact cells
lysate
lysate
kDa
50
37
α-V5
α-p24
trypsin – – + + – + + – + +
TX100 – – – + – – + – – +

Fig. 14


A
SMS1
SMS2
SMSr
1.9-
1.2-
0.8-
B
SMS1 siRNA
Lamin A siRNA
SMS2 siRNA
αLamin A
αSMS1-V5
C
αGM130
αSMS1-V5
Lamin A siRNA
SMS1 siRNA
D
Cell number (x10³)
80
60
40
20
0
0
20
40
60
80
time (hrs)
Lamin A siRNA
SMS1 siRNA
SMS2 siRNA
SMS1/SMS2 siRNA

THE WORLD BANK (GSFTD)

INTERNATIONAL SEARCH REPORT

Int[illegible]onal Application No
PCT/NL2004/000488

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/12 C12Q1/48 C12N15/54 C12N5/10 C12P7/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, BIOSIS, WPI Data, PAJ, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq 'Online! 22 October 2001 (2001-10-22), "Human polypeptide SEQ ID NO 3085." XP002325874 retrieved from EBI accession no. GSP:AAM39940 Database accession no. AAM39940 see sequence -& WO 01/53312 A (HYSEQ, INC; TANG, Y., TOM; LIU, CHENGHUA; ASUNDI, VINOD; CHEN, RUI-HON) 26 July 2001 (2001-07-26) ----- -/--	1,2,4, 47,48

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2005	08. 08. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bassias, I

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq 'Online! 6 September 2002 (2002-09-06), "Human cytochrome constitutive protein 45." XP002325875 retrieved from EBI accession no. GSN:ABB09578 Database accession no. ABB09578 see sequence -& WO 02/48356 A (BIOWINDOW GENE DEVELOPMENT INC. SHANGHAI; MAO, YUMIN; XIE, YI) 20 June 2002 (2002-06-20) see SEQ ID NOs: 1 and 2 abstract	1,2,4, 47,48
X	DATABASE Geneseq 'Online! 10 February 2003 (2003-02-10), "Human secretory polypeptide SPTM SEQ ID NO 835." XP002325876 retrieved from EBI accession no. GSN:ABP75651 Database accession no. ABP75651 see sequence -& WO 02/083876 A (INCYTE GENOMICS, INC; DAFFO, ABEL; JONES, ANISSA, L; TRAN, ALANNA-PHUN) 24 October 2002 (2002-10-24) see in particular parts referring to human secretory polypetide SPTM, SEQ ID NO: 835, claim 27	1,2,4, 47,48, 54,55
X	DATABASE Geneseq 'Online! 18 December 2003 (2003-12-18), "REMAP protein #18." XP002325877 retrieved from EBI accession no. GSN:ADC42858 Database accession no. ADC42858 see sequence -& WO 03/027228 A (INCYTE GENOMICS, INC; LAL, PREETI, G; HONCHELL, CYNTHIA, D) 3 April 2003 (2003-04-03) see in particular parts referring to REMAP protein #18, claim 1, SEQ ID NO: 18	1,2,4,7, 47,48
X	DATABASE Geneseq 'Online! 1 October 2001 (2001-10-01), "Human bone marrow protein, SEQ ID NO: 346." XP002325878 retrieved from EBI accession no. GSN:AAM00870 Database accession no. AAM00870 see sequence	1,2,7, 47,48

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Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	& WO 01/53453 A (HYSEQ, INC; FORD, JOHN, E; BOYLE, BRYAN, J; TANG, Y., TOM; LIU, CHENGH) 26 July 2001 (2001-07-26) see in particular parts referring to SEQ ID NO: 346 and claim 10	
X	DATABASE EPO Proteins 'Online! 2 February 2004 (2004-02-02), "Sequence 8703 from Patent WO0171042." XP002325879 retrieved from EBI accession no. EPOP:CQ580945 Database accession no. CQ580945 see sequence -& WO 01/71042 A (PE CORPORATION) 27 September 2001 (2001-09-27) see in particular parts referring to sequence 8703	1,2,7, 47,48
P,X	HUITEMA KLAZIEN ET AL: "Identification of a family of animal sphingomyelin synthases." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 23, no. 1, 14 January 2004 (2004-01-14), pages 33-44, XP002325870 ISSN: 0261-4189 the whole document	1-35, 38-63
P,X	WO 03/073826 A (SAGRES DISCOVERY; MORRIS, DAVID, W) 12 September 2003 (2003-09-12) see the whole document, in particular claim 5, parts referring to mCP2702 and SEQ ID NO: 291 -& DATABASE Geneseq 'Online! 18 November 2004 (2004-11-18), "Mouse protein sequence mCP2702." XP002325880 retrieved from EBI accession no. GSN:ABM85292 Database accession no. ABM85292 see sequence	1,2,4, 47,48
P,X	DATABASE Geneseq 'Online! 15 January 2004 (2004-01-15), "Human NOV13a SEQ ID 54." XP002325881 retrieved from EBI accession no. GSN:ADD49081 Database accession no. ADD49081 see sequence -/--	1,2,4, 47,48

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL2004/000488

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	-& WO 03/060149 A (CURAGEN CORPORATION; GROSSE, WILLIAM, M; ALSOBROOK, II, JOHN, P; ANDER) 24 July 2003 (2003-07-24) see parts relating to NOV13a, SEQ ID NO: 54; claim 1; page 167	1,2,4, 47,48
P,X	DATABASE Geneseq 'Online! 20 May 2004 (2004-05-20), "Human disease detection and treatment (MDDT) protein - SEQ ID 116." XP002325882 retrieved from EBI accession no. GSN:ADL22667 Database accession no. ADL22667 see sequence	1,2,4,7, 47,48
P,X	-& WO 03/062379 A (INCYTE GENOMICS, INC; JONES, ANISSA, L; DAHL, CHRISTOPHER, R; GIETZEN,) 31 July 2003 (2003-07-31) see in particular parts referring to MDDT protein, SEQ ID NO: 116, claim 27	1,2,4,7, 47,48
P,X	YAMAOKA SHOHEI ET AL: "Expression cloning of a human cDNA restoring sphingomyelin synthesis and cell growth in sphingomyelin synthase-defective lymphoid cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 18, 30 April 2004 (2004-04-30), pages 18688-18693, XP002325871 ISSN: 0021-9258 the whole document	1,2,4, 9-33, 38-63
P,A	LUBERTO CHIARA ET AL: "Purification, characterization, and identification of a sphingomyelin synthase from Pseudomonas aeruginosa. PlcH is a multifunctional enzyme." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 35, 29 August 2003 (2003-08-29), pages 32733-32743, XP002325872 ISSN: 0021-9258	
A	LUBERTO CHIARA ET AL: "Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation: Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 23, 5 June 1998 (1998-06-05), pages 14550-14559, XP002325873 ISSN: 0021-9258	

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INTERNATIONAL SEARCH REPORT

national application No.
PCT/NL2004/000488

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 3,5,6,8
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 3, 5, 6, 8
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-35, 38-63

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-35, 38-63

Polypeptides having the requested identity to the motifs as given in claim 1 or to the specific amino acid sequences as given in SEQ ID NOs: 12-22, with the mentioned enzymatic activity as described in claims 9-11, the corresponding nucleic acid sequences encoding said polypeptides, plasmids and microorganisms comprising said nucleic acid sequences processes and uses of the enzymes in question and the thereof produced products, methods for improving the yield of an secretion product in a cell and methods for targeting polypeptides in cellular compartments.

---

2. claims: 36, 37

Process to isolate candidates for functional genes of a previously unidentified enzyme with known activity.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Claims Nos.: 3,5,6,8

Claim 1 relates to polypeptides consisting of a sequence which has at least 80% identity to motifs which are defined mainly by variable amino acid residues. Motif (a) for example consists of 7 defined amino acid residues and 53-93 undefined amino acid residues. The requested percentage of identity is not limited to the defined part of the sequence but refers to the complete motif embracing also the undefined part of the motif. Any polypeptide falls into the scope of such a claim, since any amino acid sequence has 80% identity with the undefined part of the motif (and the resulting 20% ?non-identity? with the fixed part of the motif). Consequently, no meaningful search could be performed for such an unclear claim and thus the search for claim 1 was limited to polypeptides having 100% identity to any of the motifs (a) ? (c). Furthermore, it has to be noted that claim 1 comprises numbers within the motifs which are nowhere explained and thus render the true scope of the claim unclear (Article 6 PCT). From the specific sequences 12-22 it could be derived that ?X(35,75)? must mean 35 to 75 variable amino acid residues (?X(8)? means 8 variable amino acid residues). Furthermore, the amino acids given in square brackets '! where interpreted as possible alternatives at this position of the motif. Hence, claim 1 was searched with the above mentioned limitation and the interpretation of the unclear terms as given above.

Claim 3 refers to polypeptides having at least 70% similarity to sequences (SEQ ID NO: 1-11) with about 80% undefined amino acid residues. The requested similarity is not limited to the defined parts of the sequence but refers to the complete sequences, including the undefined sections. Hence any polypeptide having any sequence would fall into the scope of such a claim (see also argumentation given for the limited search of claim 1). Since no clear technical features existed for said claim, no meaningful search was possible.

Claim 5 referring to a polypeptide comprising an amino acid sequence with at least 20% identity to any of the SEQ ID NOs: 12-22 is so broad and thus unclear in the sense of Article 6 PCT. Polypeptides having an identity of only 20% with other polypeptides are not sufficiently supported in the description. Furthermore, it appears not very credible that polypeptides being only 20% identical to each other have the same biological function. If they have different functions, then they solve different technical problems. Due to these discrepancies, no meaningful search could be performed for claim 5.
The same applies for claim 6 and claim 8, since identities of 22% or 30% are also not considered significant.

Continuation of Box II.2

Claims Nos.: 3, 5, 6, 8

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claim 1 relates to polypeptides consisting of a sequence which has at least 80% identity to motifs which are defined mainly by variable amino acid residues. Motif (a) for example consists of 7 defined amino acid residues and 53-93 undefined amino acid residues. The requested percentage of identity is not limited to the defined part of the sequence but refers to the complete motif embracing also the undefined part of the motif. Any polypeptide falls into the scope of such a claim, since any amino acid sequence has 80% identity with the undefined part of the motif (and the resulting 20% "non-identity" with the fixed part of the motif). Consequently, no meaningful search could be performed for such an unclear claim and thus the search for claim 1 was limited to polypeptides having 100% identity to any of the motifs (a) to (c). Furthermore, it has to be noted that claim 1 comprises numbers within the motifs which are nowhere explained and which render the true scope of the claim unclear (Article 6 PCT). From the specific sequences 12-22 it could be derived that "X(35,75)" must mean 35 to 75 variable amino acid residues ("X(8)" means 8 variable amino acid residues). Furthermore, the amino acids given in square brackets '! where interpreted as possible alternatives at this position of the motif. Hence, claim 1 was searched with the above mentioned limitation and the interpretation of the unclear terms as given above.

Claim 3 refers to polypeptides having at least 70% similarity to sequences (SEQ ID NO: 1-11) with about 80% undefined amino acid residues. The requested similarity is not limited to the defined parts of the sequence but refers to the complete sequences, including the undefined sections. Hence any polypeptide having any sequence would fall into the scope of such a claim (see also argumentation given for the limited search of claim 1). Since no clear technical features existed for said claim, no meaningful search was possible.

Claim 5 referring to a polypeptide comprising an amino acid sequence with at least 20% identity to any of the SEQ ID NOs: 12-22 is so broad and thus renders the true scope of the claim unclear (Article 6 PCT). Polypeptides having an identity of only 20% with other polypeptides are not sufficiently supported in the description. Furthermore, it appears not very credible that polypeptides being only 20% identical to each other have the same biological function. If they have different functions, then they solve different technical problems. Due to these discrepancies, no meaningful search could be performed for claim 5. The same applies for claim 6 and claim 8, since identities of 22% or 30% are also not considered significant.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

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INTERNATIONAL SEARCH REPORT

ormation on patent family members

Int ional Application No
PCT/NL2004/000488

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INTERNATIONAL SEARCH REPORT

...formation on patent family members

Int ...nal Application No
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		AU 2728401 A	31-07-2001
		AU 2734401 A	31-07-2001
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